Aza-peptide Aldehydes and Ketones: A New Class of Inhibitors for Proteases

Research Thesis

Presented in partial fulfillment of the requirements for graduation
with research distinction in Chemistry* in the undergraduate
colleges of The Ohio State University

by

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April 2017

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ABSTRACT

Proteases are enzymes that catalyze the hydrolysis of specific peptide bonds in proteins, a process referred to as proteolysis. Proteases are largely responsible for processes such as apoptosis, immune response, protein turnover, digestion, blood coagulation and wound healing, fertilization, cell differentiation and growth, and cell signaling among other processes. Thus, uncontrolled proteolysis can lead to many disease states, such as cancer, neurological disorders, viral infections, inflammation, and arthritis. Aza-peptide aldehydes and ketones are a new class of inhibitors designed to inhibit the proteasome and the clan CD cysteine proteases for the potential treatment of cancer and neurodegenerative disorders. These compounds are designed starting with the structure of a good target protease substrate structure. The α-carbon of the P1 amino acid residue is replaced with a nitrogen atom to make an Aza-amino acid residue and the scissile peptide bond is replaced with an aldehyde or ketone moiety. Aza-peptides are more rigid than their peptide analogs due to the inability of the N-CO bond at the P1 site to rotate. We hypothesize that the rigidity of aza-peptides makes these inhibitors more selective, as other proteases will be unable to accommodate this rigidity while the proteasome and the clan CD cysteine proteases will be able to. Here I will present the synthesis and the kinetic inhibition study of the first examples of the proteasome and the clan CD cysteine protease specific aza-peptide aldehyde and ketone inhibitors.

Acknowledgements

I would like to thank my thesis advisor, Dr. Özlem Doğan Ekici, for the opportunities she has given me over the past few years, for her encouragement to present my research and the time she has spent helping me grow as a scientist. Furthermore, for all of the work she has put into making this project possible.

I want to thank Thomas Corrigan for always being willing to answer my questions in lab and for taking the time to teach me various research techniques and finally for all of his hard work on synthesizing and interpreting the results presented here.

I would like to thank Dr. Hadad for the funding he has given to my project.

I want to thank Dr. Ryan Yoder and his student, Ryan McCauslin for all of the computational work they did for the project.

I want to thank The Ohio State University at Newark, The Ohio State University at Marion and the Ohio Supercomputing Center (OSC) for the funding and resources that make this research possible.

Finally, I would like to thank my parents, Mark and Kathy, for supporting me in every challenge I have taken on and always believing that I could do it, my siblings, Derek, Blake, Erika and Grant for all of their help over the past years from reading papers, to helping with homework and for being there when I needed them.

TABLE OF CONTENTS

ABSTRACT…….ii

ACKNOWLEGMENTS……iii

LIST OF FIGURES……..……...v

LIST OF SCHEMES……..……vi

LIST OF TABLES…….vii

ABBREVIATIONS…….…..vii

CHAPTERS

1. INTODUCTION……..……..1

2. RESULTS AND DISSCUSSION…….……..7

2.1 Synthesis

2.2 Proteasome Inhibition……...…9

2.3 Caspase -3 Inhibition…….....12

2.4 Legumain Inhibition……...13

3. CURRENT AND FUTURE WORK……..15

3.1 Proteasome……...15

3.2 Caspases……...15

4. EXPERIMENTAL……..15

4.1 Methods……...…….15

4.2 Procedures of Synthesized Compounds……..…..16

APPENDIX A……..24

REFERENCES……..……..21

LIST OF FIGURES

Figures

Figure 1.1. Proteolysis of peptide bonds in proteins……....1

Figure 1.2. Reversible inhibitors of cysteine serine, and threonine proteases……...…..2

Figure 1.3. Irreversible inhibitors of cysteine, serine and threonine proteases……...….2

Figure 1.4. Proteasome inhibitors approved by the FDA for treatment of MM……..…3

Figure 1.5. Proteasome complex image 3-9……..4

Figure 1.6. Protease nomenclature by Schechter and Berger……..…5

Figure 1.7. Aza-peptide ketone and aldehyde inhibitor design……...5

LIST OF SCHEMES

Schemes

Scheme 2.1. General reactions of aza-peptide aldehyde and ketone inhibitors ……8

LIST OF TABLES

Tables

Table 2.1. Inhibition of the 20s proteasome by aza-peptide aldehyde and ketone inhibitors……9

Table 2.2 Synthesized aza-peptide 20s proteasome inhibitors……..10

Table 2.3 Synthesized aza-peptides aldehyde and ketones specific for caspase-3……12

Table 2.4 Synthesized caspase-3 aza-peptide aldehyde and ketone inhibitors ……...……..12

Table 2.5. Inhibition of legumain by aza-peptide aldehyde and ketone inhibitors ……...……...13

Table 2.6. Synthesized aza-peptide ketone's legumain inhibitors…….13

ABBREVIATIONS

Alanine (Ala)

Aldehyde (COH)

Asparagine (Asn)

Aspartic (Asp)

Benzyl ketone (COBn)

Carboxybenzyl (Z)

Dichloromethane (DCM)

Dimethyl sulfoxide (DMSO)

Dithiothreitol (DTT)

Glycine (Gly)

Isobutyl chloroformate (*i*BCF)

Leucine (Leu)

Methanol (MeOH)

Methyl ketone (COMe)

N,N-Diisopropylethylamine (DIPEA)

N-Methylmorpholine (NMM)

Palladium on Carbon (Pd/C)

Phenylalanine (Phe)

Polyethylene glycol (PEG)

Pyrazine (Pz)

Room temperature (rt)

Sucrose (Suc)

Tetrahydrofuran (THF)

Valine (Val)

1. INTRODUCTION

Proteases are enzymes that catalyze the hydrolysis of specific peptide bonds in proteins, a process referred to as proteolysis (Figure 1.1) [1]. To date approximately six hundred proteases have been identified, which make up ~2% of the genomes [2]. Proteases play an important role in cell processes such as apoptosis, immune response, protein turnover, digestion, blood coagulation and wound healing, fertilization, cell differentiation and growth, and cell signaling among other processes [3]. Due to their large involvement in such processes, uncontrolled proteolysis can lead to many disease states such as cancer, neurological disorders, viral infections, inflammation, and arthritis [3]. Uncontrolled proteolysis can be stopped by inhibition, thus research in design and synthesis of novel inhibitors is of interest to many researchers today [4]. While many inhibitors have been synthesized, most are nonselective and react with other proteases. Thus they cannot be considered as therapeutic agents. Hence, the challenge in protease inhibition is to come up with a design, where the electrophilic warhead is reactive enough to result in inhibition, and inert enough to be specific for the targeted protease family or clan. Proteases are categorized into families based on lineage: cysteine, serine, threonine, aspartic and metallo proteases. Clan definition is based mainly on 3-D structure/fold of the proteases within the same family. Proteases have been found to adapt to a variety of conditions, such as pH and reductive environments to name a couple [1].

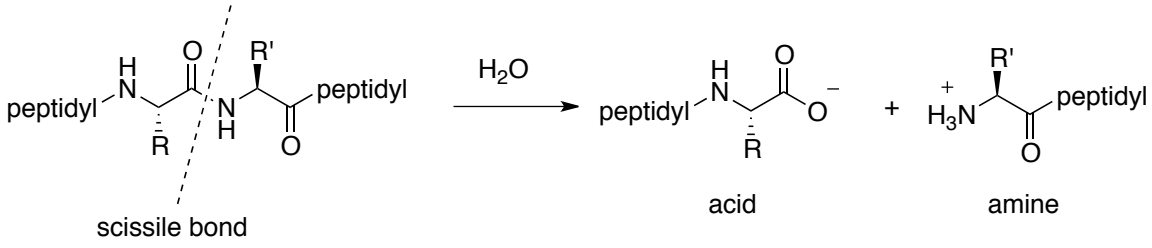


Figure 1.1. Proteolysis of peptide bonds in proteins

Successful inhibition of proteases has been achieved with both reversible inhibitors (Figure 1.2) and irreversible inhibitors (Figure 1.3). A successful inhibitor is comprised of an ideal peptide chain that is recognized by its target protease that is attached to an electrophilic group for inhibition.

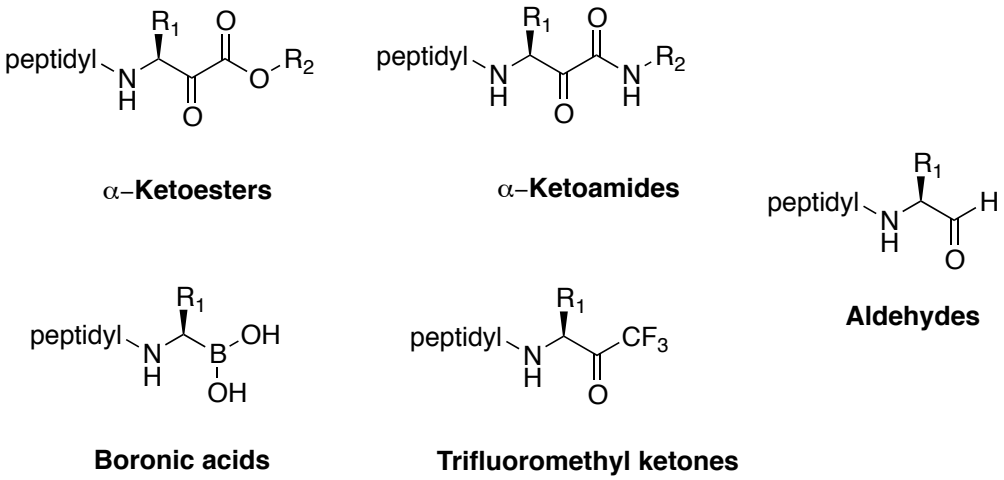


Figure 1.2. Reversible inhibitors of cysteine serine, and threonine proteases

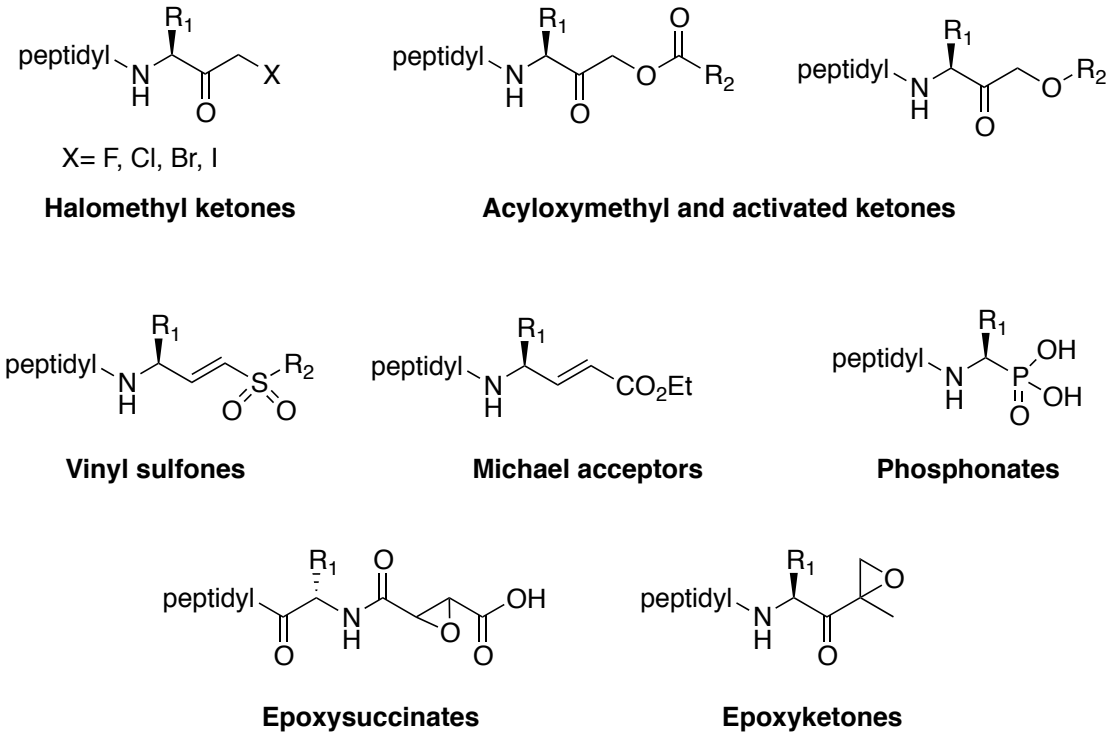
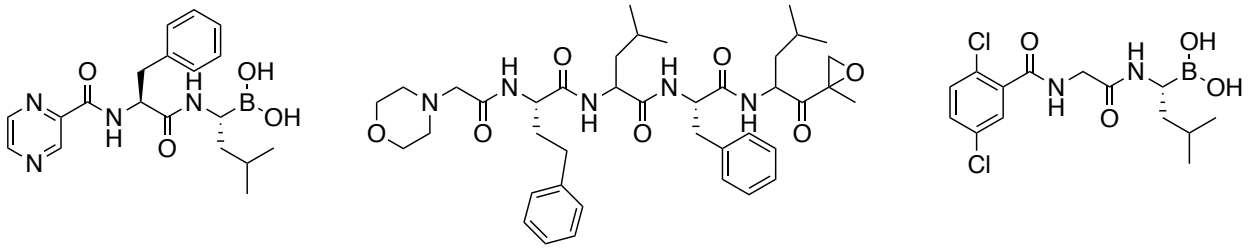


Figure 1.3. Irreversible inhibitors of cysteine, serine and threonine proteases

One specific target, the 20S proteasome will be further explored here. Proteasome is a threonine protease, which degrades proteins by the ubiquitin proteasome pathway (UPP) [1, 5]. Uncontrolled proteolysis is observed in cancers such as Multiple Melanoma (MM) and Non-Hodgkin's Lymphoma (NHL) [6, 7]. Inhibition of proteasome leads to death of cancerous cells [8, 9]. Proteasomes involvement in MM and NHL make it an interest to investigators in the field. Currently three proteasome inhibitors are on the market, however, due to unwanted side effects such as peripheral neuropathy, fatigue, generalized weakness, nausea, diarrhea, vomiting and poor appetite, infective results and drug resistance, new inhibitors are still needed (Figure 1.4) [10, 11, 12].



Bortezomib **Carfilzomib** **Ixazomib**

Figure 1.4. Proteasome inhibitors approved by the Food and Drug Administration (FDA) for treatment of MM

The proteasome complex is comprised of the 20S proteasome core, which consist of two inner β subunits and two outer α subunits, with two 19S regulatory caps at either end, which regulate entry of proteins into the active sites (Figure 1.5). There are three active sites within the proteasome: chymotrypsin-like, trypsin-like and caspase-like [13].

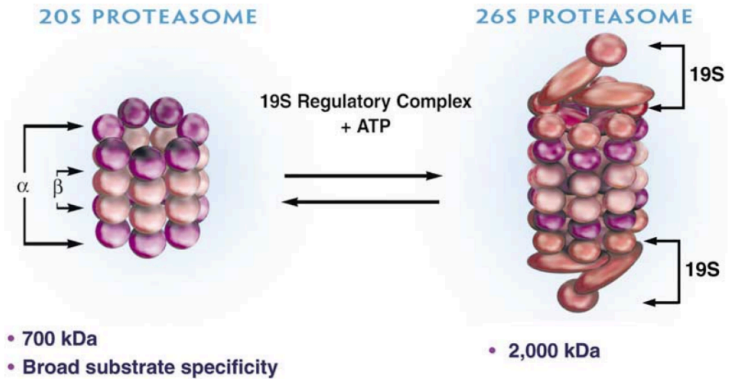


Figure 1.5. Proteasome complex image [12]

Clan CD cysteine proteases, specifically caspase-3 and legumain inhibitors, will also be further explored here. Clan CD cysteine proteases are distinctive to other classes of proteases, because of their unique α/β fold; furthermore this clan shows high specificity at the P1 site [14]. Cysteine proteases have been discovered in bacteria, protozoa, fungi, plant viruses and mammals [15]. Hydrolysis by clan CD cysteine proteases involves catalytic attack of a nucleophilic thiol in the active site.

Caspases are a part of a family of more than 15 members, 11 of these members have been identified in humans [16.] Members of this family have been identified as playing key roles in inflammation and apoptosis [17]. Caspase-3 is involved in neuronal apoptosis; uncontrolled apoptosis can cause neurological disorders such as Alzheimer's disease, spinal muscular atrophy, Parkinson's disease, Huntington's disease, Amyotropic Lateral Sclerosis (ALS) and Multiple Sclerosis (MS) [18]. Caspase's involvement in these diseases has made it a target of interest, however, there are currently no caspase inhibitors in the drug market available for the treatment of such diseases.

The second clan CD cysteine protease of interest is legumain. To date legumain has been identified in mammals, parasites and plants. Legumain has been identified as an important component of the life cycle of a parasite called *S. mansoni*, which can be found in infected humans [19]. Contracting this parasite can be detrimental to human life; thus treatment is required. While treatments for this parasite are available, there are concerns about drug resistance exist. Thus researchers are compelled to discover new drugs [20]. Research has shown that inhibition of key proteases in mice affected by the parasite had a decrease in parasite activity, thus inhibition of legumain is a promising target [20]. Similar to caspase-3, there are currently no legumain inhibitors available in the drug market for such treatments.

Nomenclature

The aza-amino acid group at the P1 site will be referred to as "A" followed by the abbreviated amino acid name. Figure 1.6 will be referred to for site specificity for proteasome, caspase-3 and legumain as well as the design of our inhibitor.

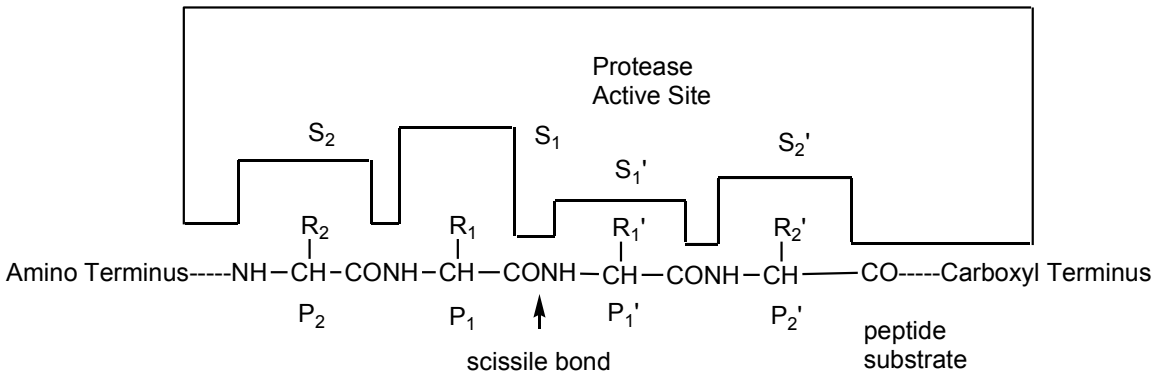


Figure 1.6. Protease nomenclature by Schechter and Berger [21]

Inhibitor Design

Aza-peptide aldehydes' and ketones' design starts with the structure of a good target protease substrate. The α-carbon of the P1 amino acid residue is replaced with a nitrogen atom to make an aza-amino acid residue and the scissile peptide bond is replaced with an aldehyde or ketone moiety (Figure. 1.7). Aza-peptides are more rigid than their peptide analogs due to the inability of the N-CO bond at the P1 site to rotate. Aza-peptide inhibitor design for proteasome, caspase and legumain is shown below in Figure 1.7.

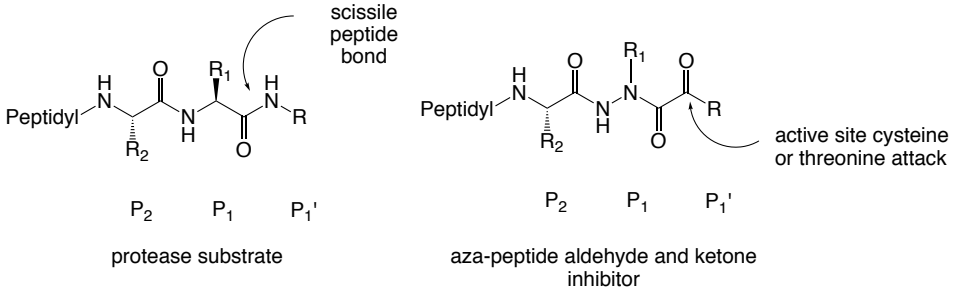


Figure 1.7. Aza-peptide aldehyde and ketone inhibitor design

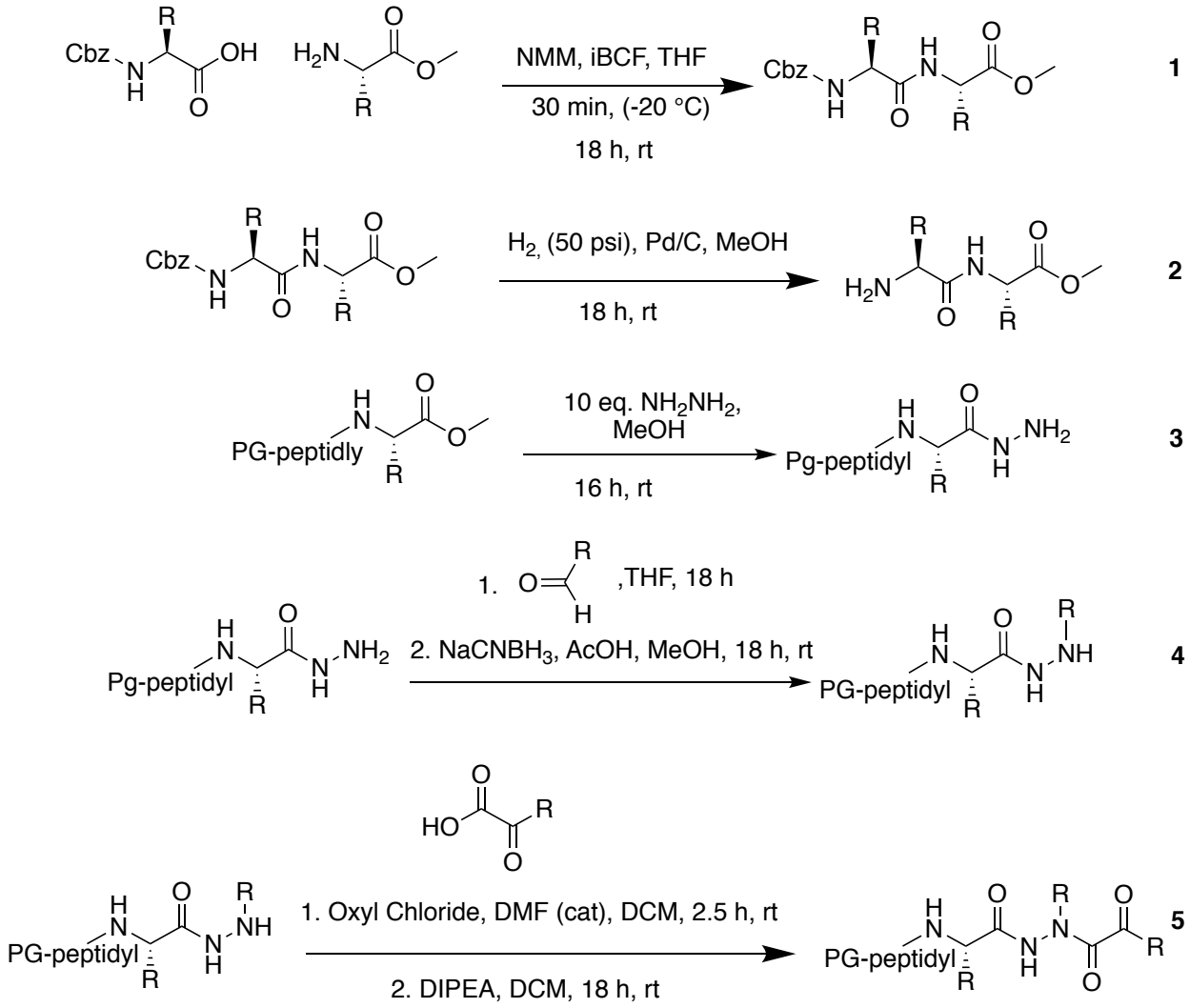
We hypothesize that the rigidity of aza- peptides makes these inhibitors more selective, as other proteases will be unable to accommodate this rigidity while the proteasome and the clan CD cysteine proteases will be able to. Past research on caspase and legumain inhibitors has shown that this clan of cysteine protease tolerates aza-peptides [15, 22].

We expect our compounds to be reversible covalent inhibitors. Reversible covalent inhibitors are believed to be less toxic than their counterparts, because they often do not reach sufficient levels to induce an immune response, making them desirable drug candidates [23].

2. Results and Discussion

2.1 Synthesis

Peptides **2** were synthesized by general peptide coupling procedure where a protected amino acid was coupled to either an amino methyl ester or peptidyl methyl ester by reacting with *i*BCF and NMM in THF overnight at rt. Peptidyl hydrazides were synthesized by reacting methyl ester with hydrazine in methanol overnight at rt **3**. Hydrogenation to deprotect peptides was done by reacting a protected peptide with hydrogen, palladium on carbon in methanol at rt overnight. Substituted peptidyl hydrazides were synthesized by reductive amination of peptidyl hydrazide with sodium cyanoborohydride, catalytic amounts of acetic acid and an aldehyde in methanol overnight at rt. Substitution of carbonyl chloride by substituted peptidyl hydrazide gave aza-peptide ketones' and aldehydes' **5.** Aza-peptide ketones and aldehydes were synthesized by reacting oxyl chloride with carboxyl acid derivatives and catalytic amounts of DMF in DCM under argon at rt for 2.5 h then concentrated. Concentrated product was reacted with substituted peptidyl hydrazides and DIPEA under argon in DCM overnight at rt. The majority of the final compounds were synthesized and characterized by Thomas Corrigan.



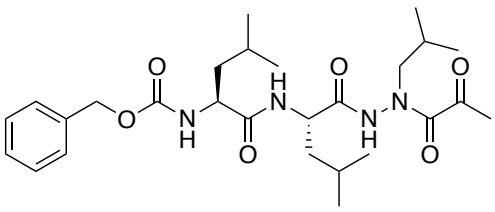
Scheme 2.1. General reactions of aza-peptide aldehyde and ketone inhibitors

2.2 Proteasome Inhibitors.

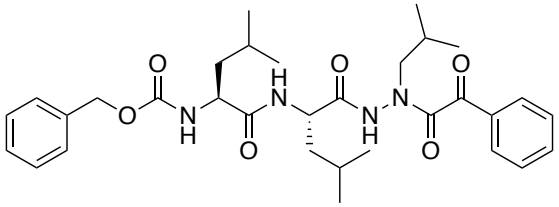
PG	P3	P2	P1	Warhead	K_i (μM)
Z	Leu	Leu	ALeu	COMe	27.39 ± 7.0
Z	Leu	Leu	ALeu	COBn	81.10 ± 15.8
Z	Leu	Leu	ALeu	COH	23.84 ± 8.1
Z	Leu	Phe	ALeu	COMe	46.67 ± 19.5
Z	Leu	Phe	ALeu	COBn	54.78 ± 13.7
Z	Leu	Phe	ALeu	COH	Not tested
Z	Leu	Leu	AGly	COMe	29.36 ± 6.9
Z	Leu	Leu	AGly	COH	34.88 ±5.2
Z	Leu	Leu	AAla	COMe	73.23 ± 13.6
Z	Leu	Leu	AAla	COH	44.01 ± 8.1
Pz	-	Phe	ALeu	COMe	474 ± 343
Pz	-	Phe	ALeu	COBn	Not tested
[a]Z	Leu	Leu	Leu	COH	0.0142 ± 0.003

[a]: the commercial inhibitor MG132

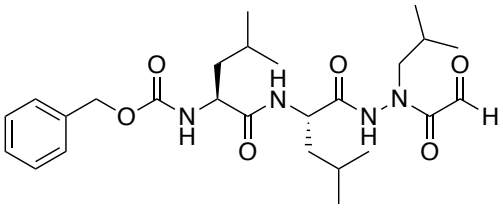
Table 2.1. Inhibition of the 20S proteasome by aza-peptide aldehyde and ketone inhibitors



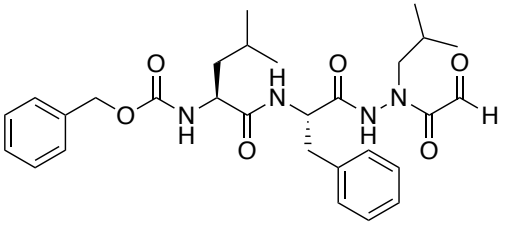
Z-Leu-Leu-ALeu-COMe



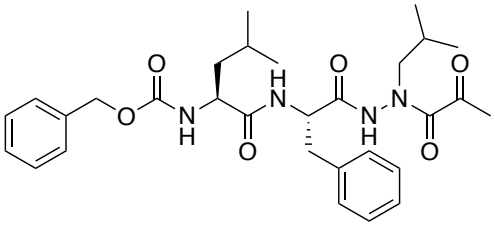
Z-Leu-Leu-ALeu-COBn



Z-Leu-Leu-ALeu-COH



Z-Leu-Phe-ALeu-COH



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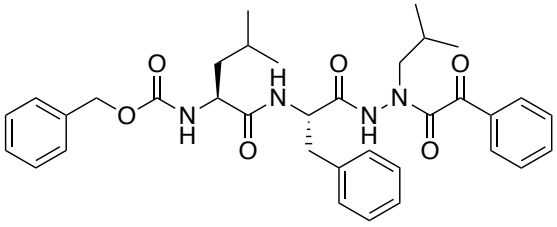
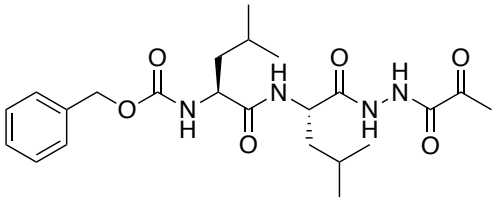
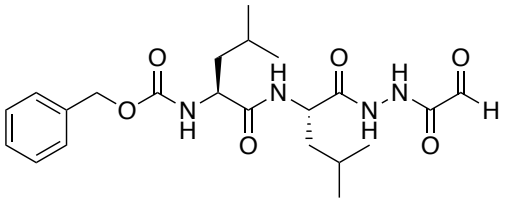


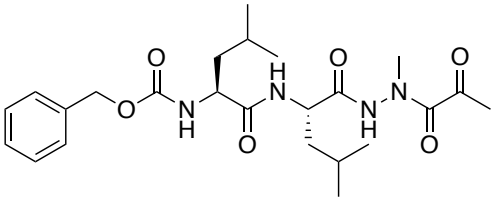
Z-Leu-Phe-ALeu-COBn



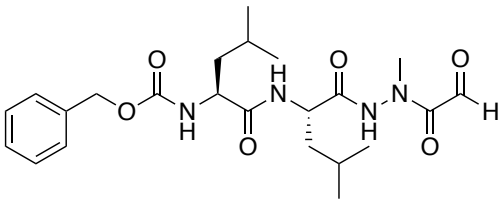
Z-Leu-Leu-AGly-COMe



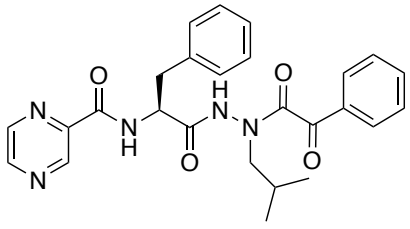
Z-Leu-Leu-AGly-COH



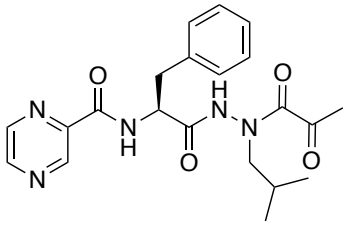
Z-Leu-Leu-AAla-COMe



Z-Leu-Leu-AAla-COH



Pz-Phe-ALeu-COBn



Pz-Phe-ALeu-COMe

Table 2.2 Synthesized aza-peptide 20S proteasome inhibitors

Aza-peptide aldehyde and ketone compounds are found to be potent inhibitors of proteasome. Molecular docking of α-ketohydrazide in the β5 subunit showed a distance of 2.94 Å between the electrophilic warhead and oxygen of the catalytic threonine, which supports the hypothesized mechanism of action, nucleophilic attach of the warhead by $^{-}$O-Thr. Our inhibitor design is based on specificity of the chymotrypsin-like active site. Chymotrypsin-like active site prefers tripeptides that are non-charged and hydrophobic [24]. A variety of aza-peptides and warheads were synthesized, then tested against the 20S proteasome; a commercially sold inhibitor (MG 132) was also tested for comparison. The inhibitor constant for the synthesized inhibitors is listed in Table 2.1. Our aza-peptide aldehyde and ketone inhibitors have K_i values in the lower micro M range. Our most potent inhibitor is Z-Leu-Leu-ALeu- COH. For all of the inhibitors the methyl ketone and aldehyde warhead, were more potent than the benzyl ketone. This could be explained by the bulkiness of the benzyl group, making nucleophilic attack more difficult. The aldehyde outperformed the methyl ketone for two of the three tested sequences. Analysis of the aza-peptide sequence Z-Leu-Leu-AAla-COR showed a decrease in potency for all of the warheads attached to it, thus it can be concluded that at the P1 site AAla is not as favored over ALeu or AGly at P1. Replacement of Leu at the P2 position with Phe showed a decrease in potency for the methyl ketone, however, potency for benzyl ketone increased. Replacement of ALeu at the P1 position with AGly resulted in the methyl ketone being more potent than the aldehyde. Our least potent inhibitor was Pz-Phe-ALeu-COBn that was synthesized in part as a comparison to bortezomib (Figure 1.4). The decrease in strength of this inhibitor is not entirely unexpected given the active site's preference for tripeptides. Our specific inhibitors were tested for cross reactivity with the clan CA cysteine protease cathepsin B. Kinetic results showed no inhibition of cathepsin B.

2.3 Caspase-3 Inhibitors

PG	P4	P3	P2	P1	Warhead	IC_{50} (μM)
Z	Asp	Glu	Val	AAsp	COH	Not tested
Z	Asp	Glu	Val	AAsp	COMe	Not tested
Z	Asp	Glu	Leu	AAsp	COBn	Not tested
Z	Asp	Glu	Leu	Asp	COH	Not tested

Table 2.3. Synthesized aza-peptides aldehyde and ketones specific for caspase-3

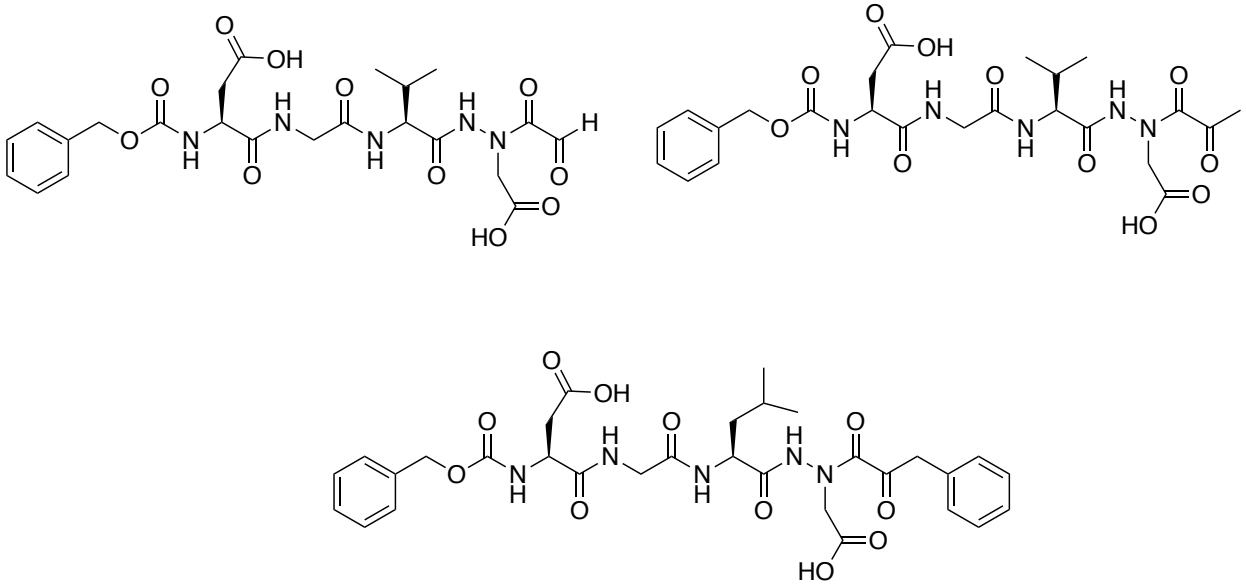


Table 2.4. Synthesized caspase-3 aza-peptide aldehyde and ketone inhibitors

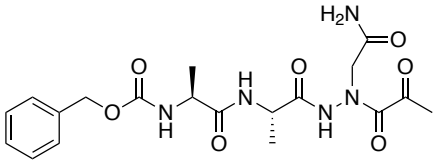
Aza-peptide aldehyde and ketone compounds targeting caspase-3 have been synthesized, however, not tested against caspase-3 at this time. Caspase 3 has a preference for tetra peptides where at the P4 site Asp is preferred likewise at the P3, P2 and P1 site Glu, Val and Asp is preferred (Figure 1.6) [25]. Our inhibitor design is based on this preference. Kinetic studies of Z-Asp-Glu-Val-AAsp-COMe with proteasome and papain reveal weak inhibition of proteasome

and no inhibition of papain. This is somewhat surprising because proteasome prefers hydrophobic peptides and the inhibitor tested against it possesses two hydrophilic groups. Additionally, papain is less specific in preference than proteasome, only having a preference that at the P2 site the residue be hydrophobic and bulky, however, unlike proteasome there was no inhibition observed.

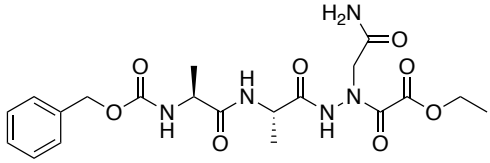
2.4 Legumain Inhibitors

PG	P3	P2	P1	Warhead	IC_{50} (µM)
Z	Ala	Ala	AAsn	COMe	Weak
Z	Ala	Ala	AAsn	COOEt	Weak
Z	Ala	Ala	AAsn	COBn	Weak

Table 2.5. Inhibition of legumain by aza-peptide aldehyde and ketone inhibitors



Z-Ala-Ala-AAsn-COMe



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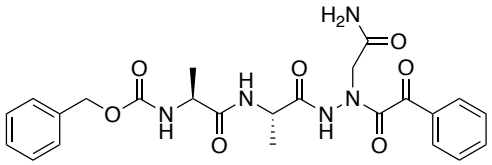

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Table 2.6. Synthesized aza-peptide ketone's legumain inhibitors

Kinetic studies of our inhibitors tested against legumain resulted in weak inhibition (Table 2.5). Our design was based on peptide specificity of schistosome legumain, the P1 site shows a preference for Asn likewise the P2 has a preference for Ala and P3 has a preference for Thr followed by Ala [26]. Previous inhibition studies with aza-peptide epoxides show potent inhibition of legumain, therefore weak inhibition cannot be attributed to the rigidity of the aza-peptide [27]. Weak inhibition must therefore be a result due to the difference in the electrophilic warhead and not the aza-peptide that it is attached to.

3. Current and Future Work

3.1 Proteasome. Current work is focused on synthesizing tetrapeptide analogs of Carfilzomib (Figure 1.4) where at the P1 site Leu is replaced with ALeu, at the P2 site Phe is replaced with Leu and the protecting group Z at the N-terminal is replaced with morpholine, these modifications are expected to increase potency. A variety of warheads including the ones presented here will be attached to that peptide sequence. In the future, we plan to test our inhibitors in vivo with cancer cell lines with collaborators at OSU Medical Center.

3.2 Caspase. Future work is focused on synthesizing and testing aza-peptide ketone and aldehyde inhibitors for clan CD cysteine proteases caspase -6 and -8.

4. Experimental

4.1 Methods. All reagents were purchased from Sigma Aldrich, Oakwood Chemical, Bachem or ApexBio and used without further purification. Dry THF was obtained from the solvent system

in the department of chemistry and used for all peptide couplings. Compounds were purified by column chromatography as necessary. Final products were verified by ^{1}H NMR and ESI.

Procedure was written and performed by Thomas Corrigan a chemistry graduate student, and Dr. Özlem Doğan Ekici at The Ohio State University. Proteasome kinetic assay details: Human 20S proteasome, 700 kDa was purchased from Boston Biochem (50 μg, 2 μM (1.4 mg/mL)) in 50 mM HEPES buffer pH 7.6, 100 mM NaCl, 1 mM DTT. Assay buffer was prepared as follows: 20 mM HEPES buffer pH 7.8, 0.5 mM EDTA, 0.037% SDS. Proteasome specific substrate Suc-LLVY-AMC was purchased from Boston Biochem and used as fluorogenic substrate; λ_{ex} = 380 nm, λ_{em} = 442 nm. Assay Protocol: 18 μL of 2 μM proteasome solution was diluted with 425 μL H_2O to make 0.06 μM stock enzyme solution for kinetic assays. In a 96-well plate suitable for fluorometric assay was added 86 μL assay buffer, 2 μL inhibitor, 2 μL enzyme substrate, and 10 μL of 0.06 μM enzyme stock solution (0.06 nM enzyme concentration in well). Fluorescence at 442 nM was monitored for 10 min. The enzyme activity was measured at varying concentrations of inhibitor (0, 25, 50, 100 μM in DMSO) and substrate (10, 20, 50, 100 μM in DMSO) by converting the slope of the plot of fluorescence intensity at 442 nm vs time for time points between 4 and 8 min using Lambert-Beer's law. K_i values were obtained from non-linear fitting of the data to a competitive inhibition model using GraphPad Prism 4.0 software.

Legumain kinetic assay details. Activation: same-pichia powder- was diluted in water. IrAE solution in water. 3 h at rt; activation in activation buffer: 0.1 M Na-Ac pH 4.0;5 mM DTT. Assay buffer: 0.1 M Na-Ac pH 5.5; 2.5 mM DTT; 0.1 % PEG 6000. Substrate: Z-AAN-AMC; 50 μM final in assay. Inhibitors in DMSO; (1 % DMSO in activity assay) incubation period 10 min at rt.

4.2 Procedures of Synthesized Compounds.

Z-Leu-Leu-OMe. Z-Leu-OH (1 eq) was dissolved in dry THF and cooled to -20 °C. NMM (1 eq) and *i*BCF (1 eq) were added dropwise and the mixture was allowed to react for 30 min. H-Leu-OMe (1 eq) was dissolved in dry THF and cooled to -20 °C. NMM (1 eq) were added dropwise and the mixture was allowed to react for 15 min. The two mixtures above were added together. The combined mixture was stirred for 1 h at -20 °C and was allowed to react for 18 h at room temperature. The solvent was evaporated and the residue was treated with EtOAc and H_2O. The organic layer was washed with 1 M HCl, H_2O, salted $NaHCO_3$, and salted NaCl, dried over Na_2SO_4, filtered and concentrated to give Z-Leu-Leu-OMe as a white in 90% yield. ^{1}H NMR (DMSO-d_6): 1.18 (m, 12 H, $(CH_3)_2CH$), 1.47 (m, 2 H, $(CH_3)_2CH$), 1.56 (m, 4 H, $(CH_3)_2CHCH_2$), 3.59 (s, 3 H, OCH_3), 4.03 (m, 1 H, $NCHCH_2C$), 4.27 (t, 1 H, $NCHCH_2C$), 5.00 (s, 2 H, CH_2Ph), 7.35 (m, 5 H, Ph), 8.19 (d, 1 H, NH).

Z-Ala-Ala-OMe. See procedure and workup of Z-Leu-Leu-OMe. A white solid in 83% yield was obtained. ^{1}H NMR (DMSO-d_6): 1.21 (d, 3 H, CH_3CH), 1.29 (d, 3 H, CH_3CH), 3.62 (s, 3 H, CH_3O), 4.08 (t, 1 H, CH_3CH), 4.27 (t, 1 H, CH_3CH), 5.01 (s, 2 H, CH_2Ph) 7.36 (m, 5 H, Ph), 8.26 (d, 1 H, NH).

Z-Leu-Phe-OMe. See procedure and workup of Z-Leu-Leu-OMe. A white solid in 83% yield was obtained. ^{1}H NMR (DMSO-d_6): 0.86 (q, 6 H, $(CH_3)_2CHCH_2$), 1.37 (m, 2 H, $(CH_3)_2CHCH_2$), 1.57 (m, $(CH_3)_2CHCH_2$), 2.98 (m, 2 H, $CHCH_2Ph$), 3.59 (s, 3 H, OCH_3), 4.06 (m, 1 H, NHCH), 4.47 (q, 1 H, NHCH), 5.02 (s, 2 H, $PhCH_2O$), 7.17-7.39 (m, 10 H, Ph), 8.28, (d, 1 H, NH). m, 5 H, Ph), 7.60 (d, 1 H, NH), 7.92 (d, 1 H, NH), 8.12 (d, 1 H, NH).

Z-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-OMe. See procedure and workup of Z-Leu-Leu-OMe. A White foam in 86% yield was obtained. ^{1}H NMR (DMSO-d_6): 0.88 (t, 6 H, $(CH_3)_2CH$), 1.37 (s, 9 H,

$(CH_3)_3O$), 1.40 (s, 9 H, $(CH_3)_3O$), 1.77 (m, 1 H, CH_2CH_2), 1.88 (m, 1 H, CH_2CH_2), 2.05 (q, 1 H, $(CH_3)_2CH$), 2.22 (t, 2 H, CH_2CH_2), 2.43 (dd, 1 H, $CHCH_2CO$), 2.50 (dd, 1 H, $CHCH_2CO$), 3.62 (s, 3 H, CH_3O), 4.00-4.19 (m, 2 H, NHCH), 4.37 (m, 1 H, NHCH), 5.04 (d, 2 H, CH_2Ph), 7.35 (m, 5 H, Ph), 7.60 (d, 1 H, NH), 7.92 (d, 1 H, NH), 8.12 (d, 1 H, NH).

Z-Glu(otBu)-Val-OMe. See procedure and workup of Z-Leu-Leu-OMe. Product obtained as a clear oil. ^{1}H NMR (DMSO-d_6): 0.88 (t, 6 H, $(CH_3)_2CH$), 1.39 (s, 9 H, $(CH_3)_3O$), 1.72 (m, 1 H, CH_2CH_2), 1.85 (m, 1 H, CH_2CH_2), 2.05 (q, 1 H, $(CH_3)_2CH$), 2.25 (t, 2 H, CH_2CH_2), 3.63 (s, 3 H, CH_3O), 4.16 (m, 2 H, NHCH), 5.02 (d, 2 H, CH_2Ph), 7.35 (m, 5 H, Ph), 7.42 (d, 1 H, NH), 8.10 (d, 1 H, NH).

Pz-Phe-OMe. Pyrazinecarboxylic acid (1 eq) was dissolved in DCM and cooled to -20 °C followed by the addition of EDC (1.5 eq), HOBt (1 eq), H-Phe-OMe (1 eq) and DIPEA (1.2 eq dropwise), the solution was warmed to rt and allowed to react for 18 h. The solvent was evaporated and the residue was treated with EtOAc and H_2O. The organic layer was washed with salted $NaHCO_3$, citric acid (10%), salted $NaHCO_3$, H_2O and salted NaCl, dried over Na_2SO_4, filtered and concentrated to give Pz-Phe-OMe as a yellow oil in 95% yield. ^{1}H NMR (DMSO-d_6): 3.23 (d, 2 H, CH_2Ph), 3.67 (s, 3 H, OCH_3), 4.82 (t, 1 H, NCH), 7.24 (s, 5 H, Ph), 8.75 (d, 1 H, CHNCCO), 8.89 (d, 1 H, CHCHNC), 9.08 (d, 1 H, CONH), 9.13 (s, 1 H, NCHCCO).

Pz-Phe-$NHNH_2$. Pz-Phe-OMe (1eq) was dissolved in methanol followed by the addition of hydrazine (10 eq), the solution was allowed to react for 18 h at rt. The solvent was evaporated to obtain Pz-Phe-$NHNH_2$ as yellow oil in 100% yield. ^{1}H NMR (DMSO-d_6): 3.07 (d, 2 H, $CHCH_2Ph$), 4.29 (s, 2H, NH_2), 5.48 (m, 1 H, NCH), 7.22 (t, 5 H, Ph), 8.69 (d, 1 H, CHNCCO), 8.74 (d, 1 H, CHCHNC), 8.87 (d, 1 H, CONHCH), 9.10 (s, 1 H, $NHNH_2$), 9.37 (s, 1 H, NCHCCO).

Z-Ala-Ala-NHNH$_2$. Follow procedure of Pz-Phe-NHNH$_2$. Z-Ala-Ala-NHNH$_2$ was obtained as a white solid in 100% yield. ^{1}H NMR (DMSO-d$_6$): 1.19 (d, 6 H, CHCH$_3$), 4.07 (m, 2 H, NH$_2$), 4.24 (m, 2 H, CHCH$_3$), 5.03 (s, 2 H, PhCH$_2$), 7.33 (s, 5 H, Ph), 7.43 (d, 1 H, NHCH), 7.91 (d, 1 H, NHCH), 9.06 (s, 1 H, NHNH$_2$).

Z-Leu-Leu-NHNH$_2$. Follow procedure of Pz-Phe-NHNH$_2$. Z-Leu-Leu-NHNH$_2$ was obtained as white solid in 100% yield. ^{1}H NMR (DMSO-d$_6$): 0.82-0.88 (m, 12 H, CH$_2$CH(CH$_3$)$_2$), 1.38 (m, 4 H, CH$_2$CH(CH$_3$)$_2$), 1.55 (m, 2 H, CH(CH$_3$)$_2$), 4.06 (m, 1 H, NCHCH$_2$), 4.27 (m, 1 H, NCHCH$_2$), 5.03 (s, 2 H, PhCH$_2$), 7.39 (m, 5 H, Ph), 7.44 (d, 1 H, NHCH), 7.86 (d, 1 H, NHCH), 9.18 (s, 1 H, NHNH$_2$).

Pz-Phe-NHN=CHCH(CH$_3$)$_2$. Pz-Phe-NHNH$_2$ (1 eq) was dissolved in dry THF followed by the addition of isobutyraldehyde (1.1 eq), sodium triacetoyborohydride (1.27 eq) and catalytic amounts of AcOH (dropwise). The solution was allowed to stir for 18 h at rt. The reaction was quenched with sodium bicarbonate then extracted with DCM and concentrated to obtain Pz-Phe-NHN=CHCH(CH$_3$)$_2$ as a yellow oil in 94% yield. ^{1}H NMR (DMSO-d$_6$): 1.04 (d, 3 H, CH(CH$_3$)$_2$), 1.10 (d, 3 H, CH(CH$_3$)$_2$), 3.14 (m, 2 H, CH$_2$Ph), 5.43 (m, 1 H, NCH), 7.25 (s, 5 H, Ph), 7.38 (d, 1 H, NHCH), 7.45 (d, 1 H, NCHCH(CH$_3$)$_2$), 8.76 (d, 1 H, NCH), 8.89 (d, 1 H, CHNCH), 9.12 (d, 1 H, NCHCCO), 11.22 (s, 1 H, NNH).

Z-Leu-Leu-NHN=CHCH(CH$_3$)$_2$. Follow procedure for Pz-Phe-NHN=CHCH(CH$_3$)$_2$. Z-Leu-Leu-NHN=CHCH(CH$_3$)$_2$ was concentrated to obtain a white solid in 100% yield. ^{1}H NMR (DMSO-d$_6$): 0.84 (d, 18 H, CH(CH$_3$)$_2$), 0.99 (t, 4 H, CH$_2$CH(CH$_3$)$_2$), 0.99 (t, 1 H, CH(CH$_3$)$_2$), 1.39 (t, 2 H, CH(CH$_3$)$_2$), 4.03 (m, 2 H, NHCH), 5.02 (s, 2 H, PhCH$_2$), 7.37 (m, 5 H, Ph), 7.41 (d, 1 H, NH), 7.74 (d, 1 H, NH), 7.93 (d, 1 H, NH), 10.81,11.01 (s, 1 H, N=CHCH(CH$_3$)$_2$).

Pz-Phe-NHNH-CHCH(CH_3)$_2$. Pz-Phe-NHN=CHCH(CH_3)$_2$ (1 eq) was dissolved in methanol and cooled in an ice bath followed by the addition of sodium borohydride (3 eq) and allowed to react for 36 h at rt. Product was quenched with 1 M HCl and extracted with DCM and H_2O, then concentrated and purified by column using methanol in DCM as the eluent to obtain Pz-Phe-NHNH-CHCH(CH_3)$_2$ as a yellow solid in 45% yield. ^{1}H NMR (DMSO-d_6): 0.84 (d, 3 H, CHCH_2(CH_3)$_2$), 0.85 (d, 3 H, CHCH_2(CH_3)$_2$), 2.44 (t, 2 H, CHCH_2(CH_3)$_2$), 3.09 (t, 2 H, CH_2Ph), 5.44 (m, 1 H, NCH), 7.24 (m, 5 H, Ph), 7.38 (d, 1 H, NH), 7.45 (d, 1 H, NH), 8.76 (d, 1 H, CHNC), 8.90 (d, 1 H,CHNCH), 9.13 (d, 1 H, NCHCHCO), 11.22 (s, 1 H, N=CH).

Pz-Phe-ALeu-COMe. Pyruvic acid (3.4 eq) and oxalyl chloride (3.4 eq) were reacted in DCM with catalytic amounts of DMF under argon at rt for 2.5 h. 2-oxopropanoyl chloride (3.4 eq) was concentrated then dissolved in DCM and cooled in an ice bath followed by the addition of Pz-Phe-NHNH-CHCH(CH_3)$_2$ (1 eq) and DIPEA (3.4 eq) the solution was brought to rt and was allowed to react for 18 h under argon. Product was extracted with DCM, H_2O and salted NaCl dried with sodium sulfate then purified by column using methanol and DCM as the eluent to obtain Pz-Phe-ALeu-COMe as a brown solid in 15% yield. ^{1}H NMR (CD_2Cl_2): 0.78 (d, 6 H, CH_2CH(CH_3)$_2$), 2.14 (s, 3 H, CH_3), 3.09 (q, 1 H, CH_2Ph), 3.16 (m, 2 H, NCH_2), 3.24 (q, 1 H, CH_2Ph), 4.71 (q, 1 H, NHCH), 7.20 (m, 5 H, Ph), 8.06 (d, 1 H, CHNCCO), 8.35 (d, 1 H, CHNCH), 8.47 (d, 1 H, NH), 8.73 (s, 1 H, NCHCCO), 9.30 (s, 1 H, NHN).

Pz-Phe-ALeu-COBn. Follow procedure for Pz-Phe-Aleu-COMe. To obtain Pz-Phe-ALeu-COBn as a brown solid in 1.8% yield. ^{1}H NMR (CD_2Cl_2): 0.82 (d, 6 H, CH_2CH(CH_3)$_2$), 3.11 (q, 1 H, CH_2Ph), 3.19 (m, 2 H, CH_2CH(CH_3)$_2$), 3.38 (q, 1 H, CH_2Ph), 4.00 (d, 2 H, COCH_2Ph), 4.70 (q, 1 H, HNCH), 7.21-7.35 (m, 10 H, Ph), 7.99 (d, 1 H, CHNCCO), 8.32 (d, 1 H, CHNCH), 8.52 (d, 1 H, NHCHCH_2), 8.79 (s, 1 H, NCHCCO), 9.33 (s, 1 H, NHN).

ESI:

Pz-Phe-ALeu-COBn: ($C_{27}H_{29}N_5NaO_4$) Measured 510.2114 m/z, Theoretical 510.2112 m/z

Pz-Phe-ALeu-COMe: ($C_{21}H_{25}N_5NaO_4$) Measured 434.1810 m/z, Theoretical 434.1810 m/z

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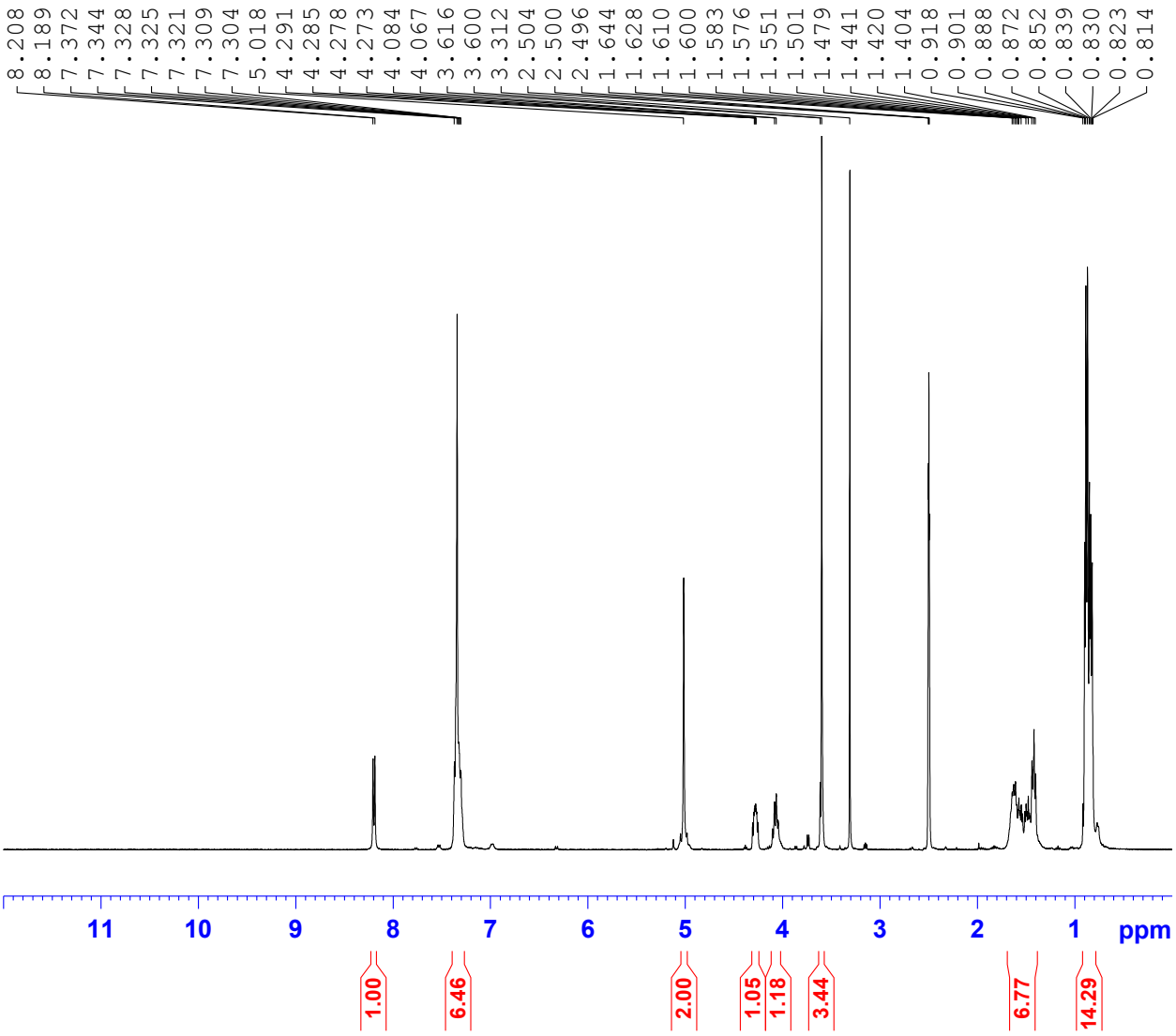
APPENDIX A

^{1}H NMR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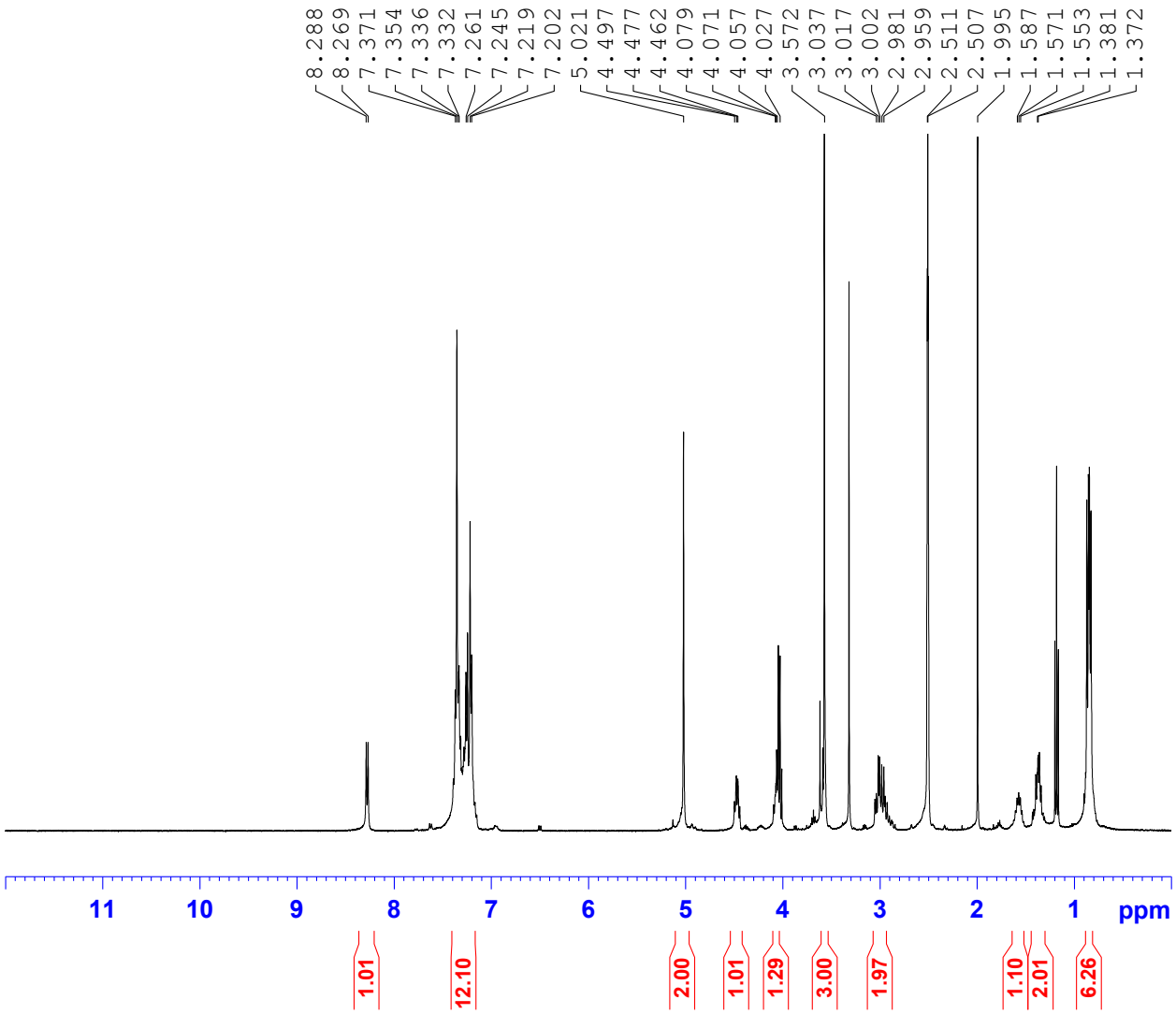
Z-Leu-Leu-OMe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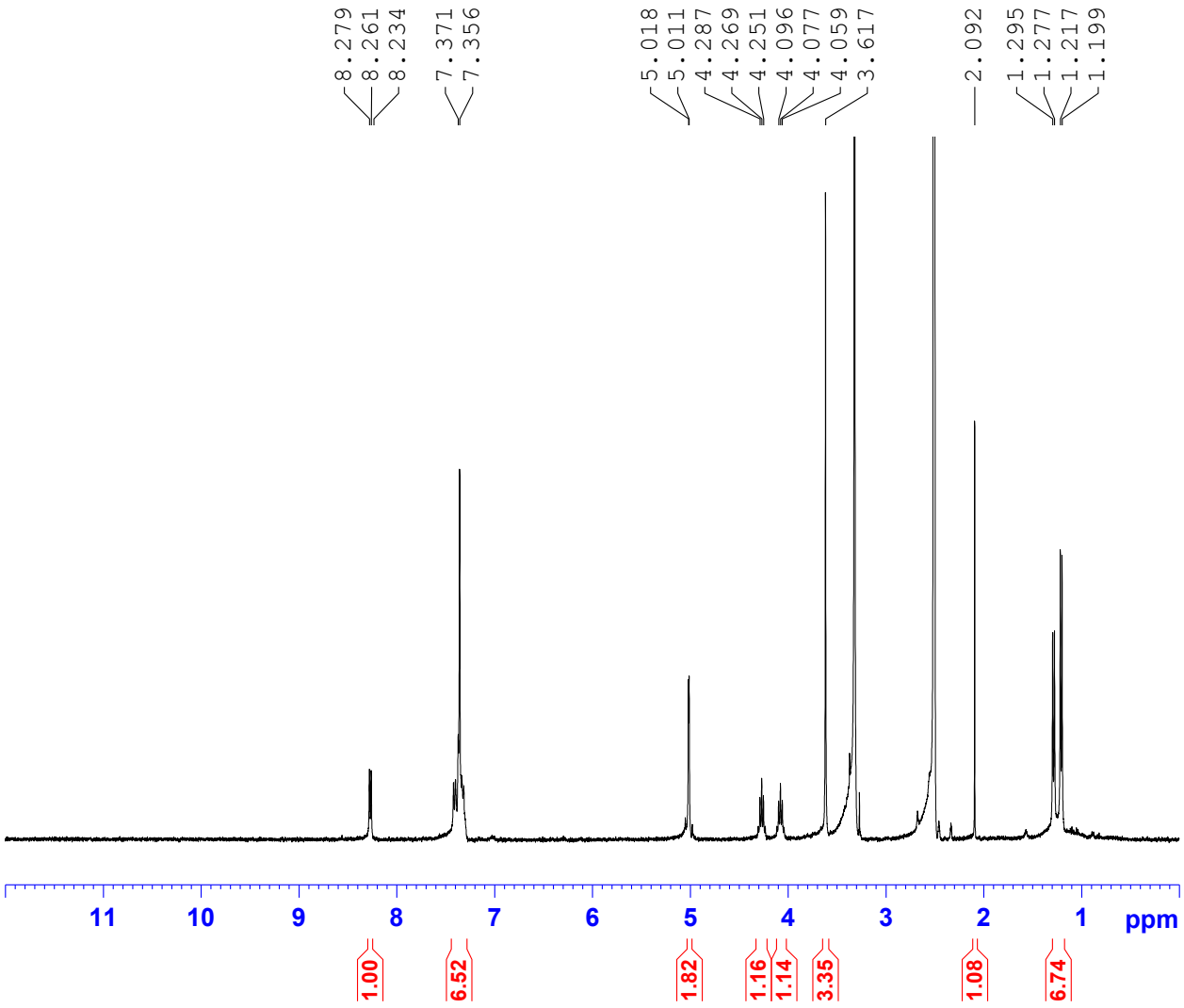
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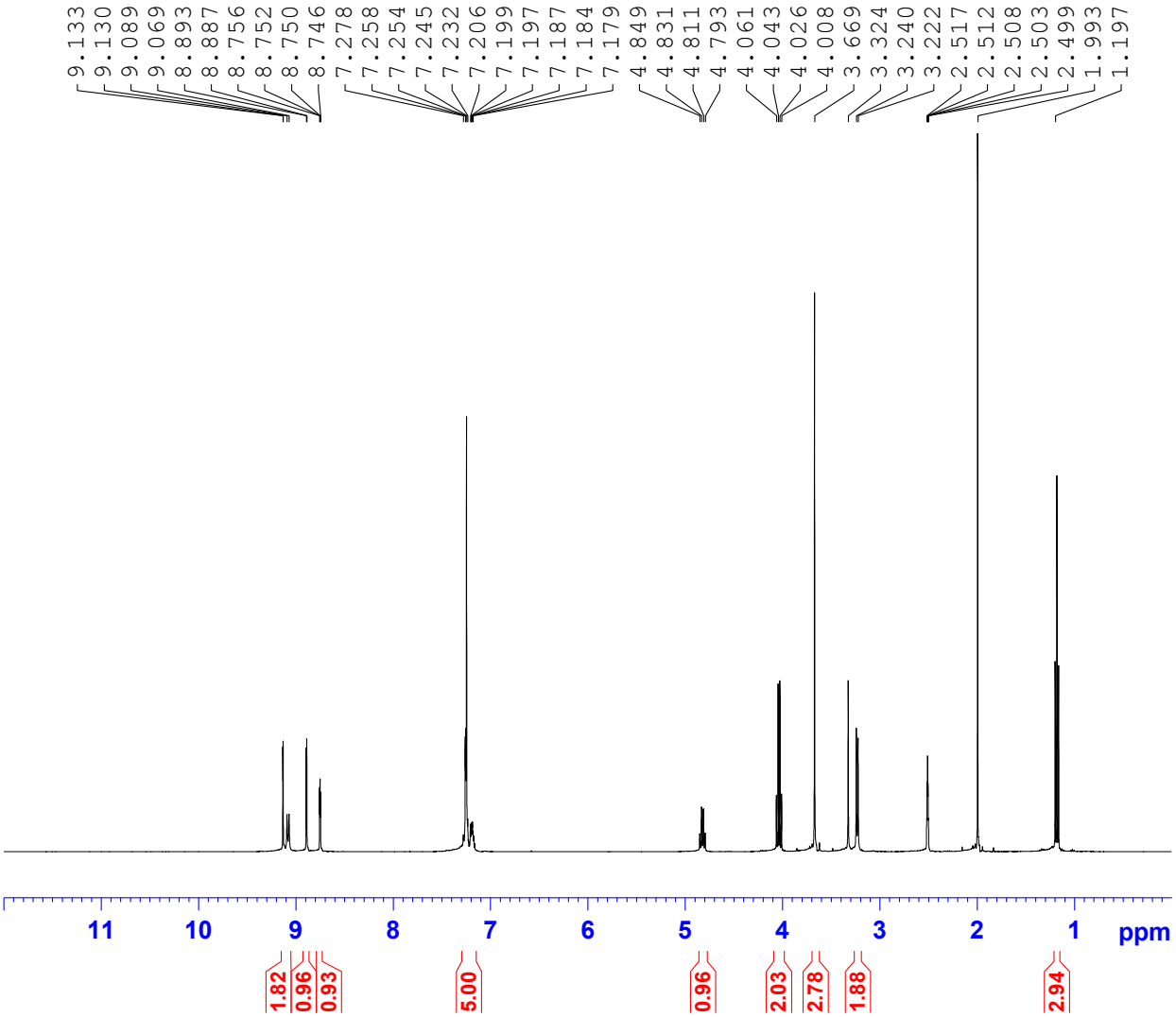
6.74

Z-Ala-Ala-OMe


9.133
9.130
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8.893
8.887
8.756
8.752
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7.278
7.258
7.254
7.245
7.232
7.206
7.199
7.197
7.187
7.184
7.179
4.849
4.831
4.811
4.793
4.061
4.043
4.026
4.008
3.669
3.324
3.240
3.222
2.517
2.512
2.508
2.503
2.499
1.993
1.197
11
10
9
8
7
6
5
4
3
2
1
ppm
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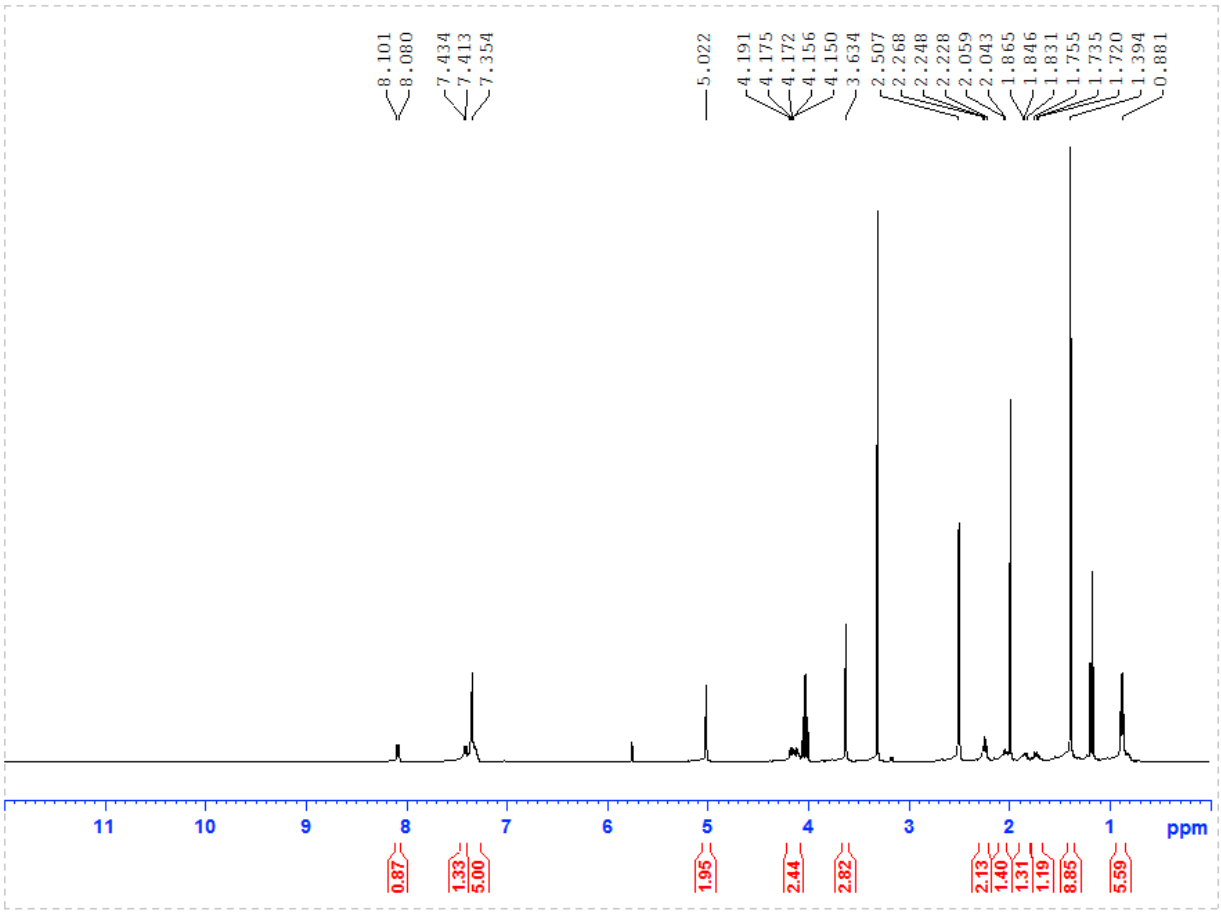
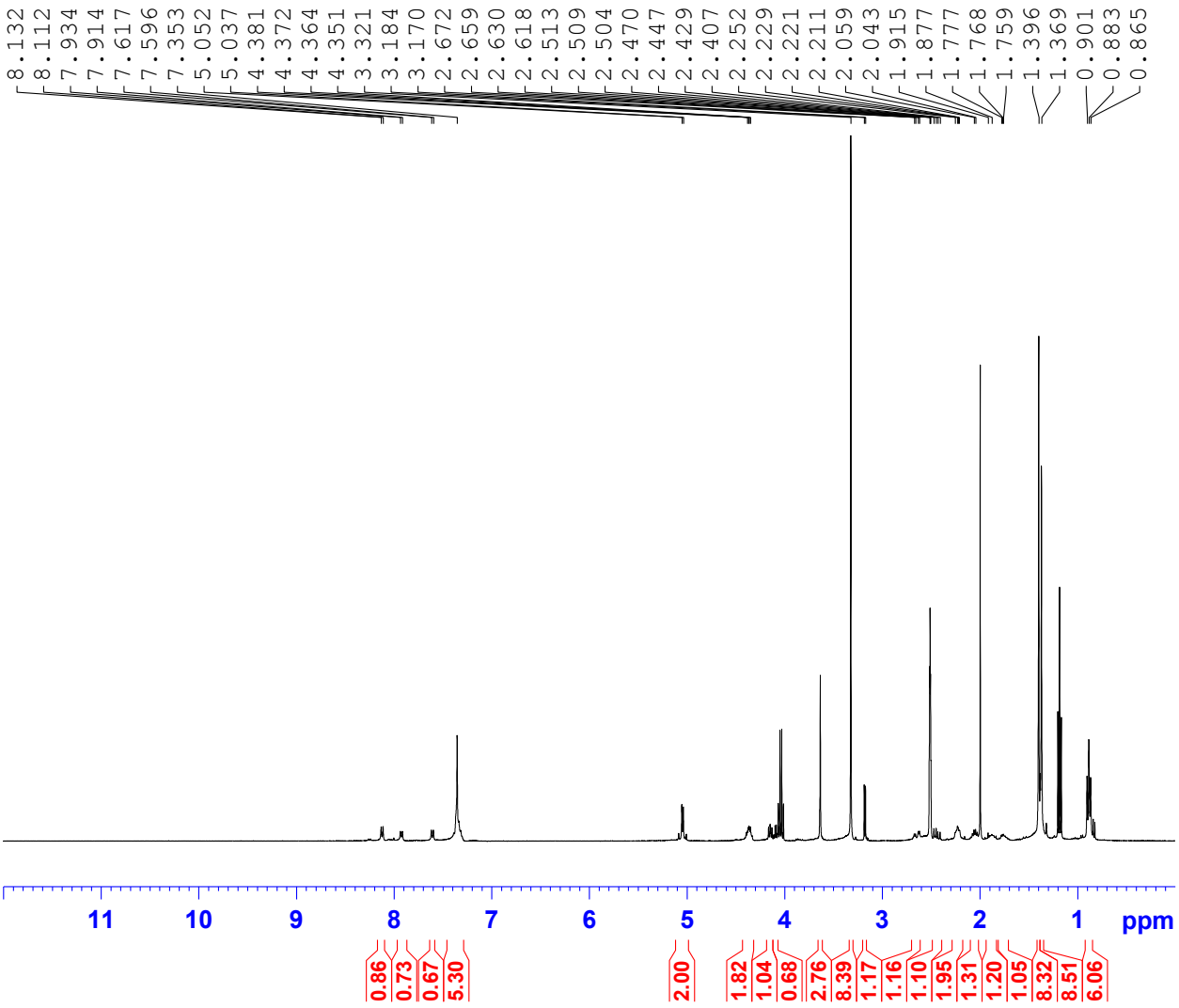
0.96
0.93
5.00
0.96
2.03
2.78
1.88
2.94

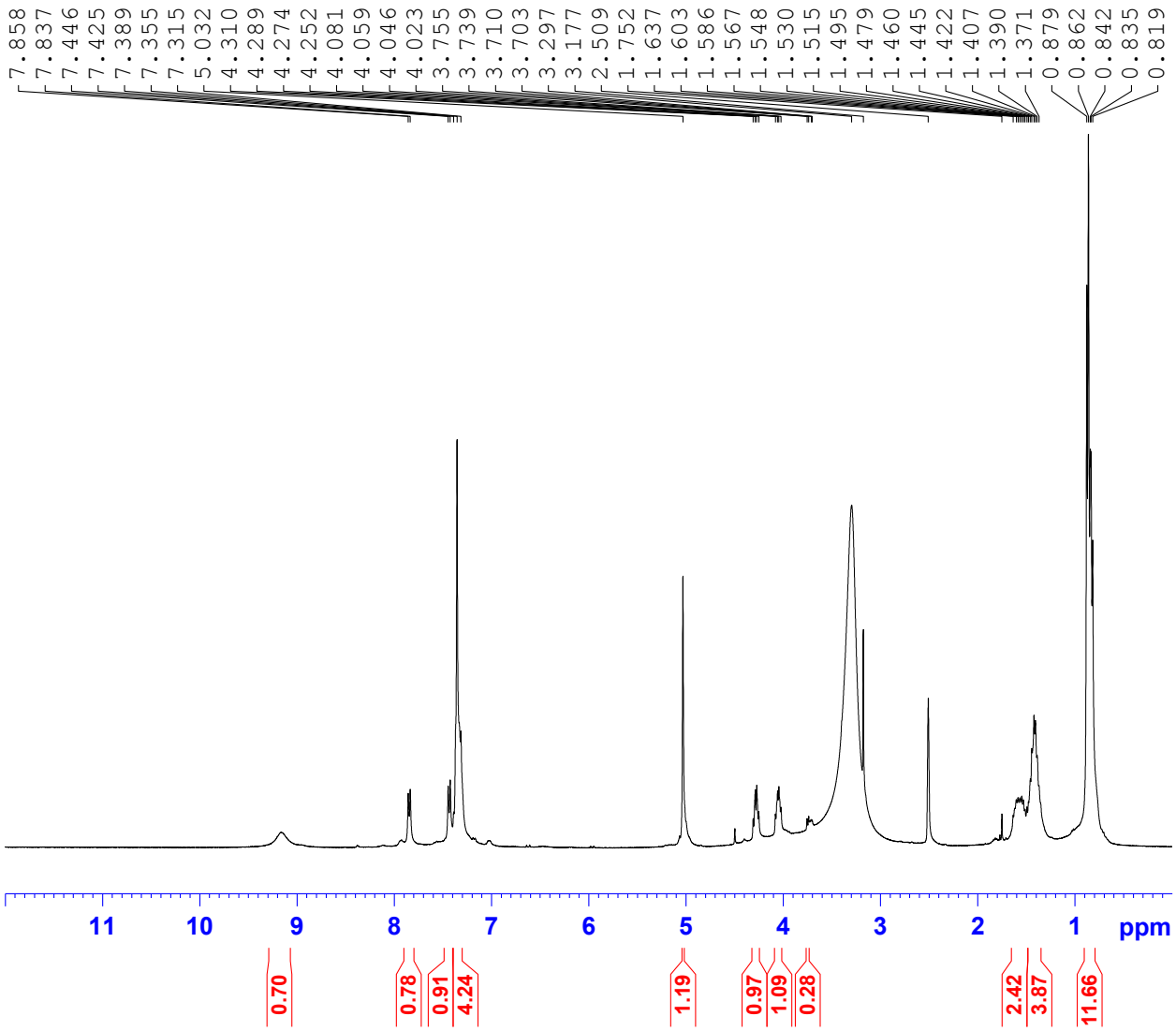
Pz-Phe-OMe



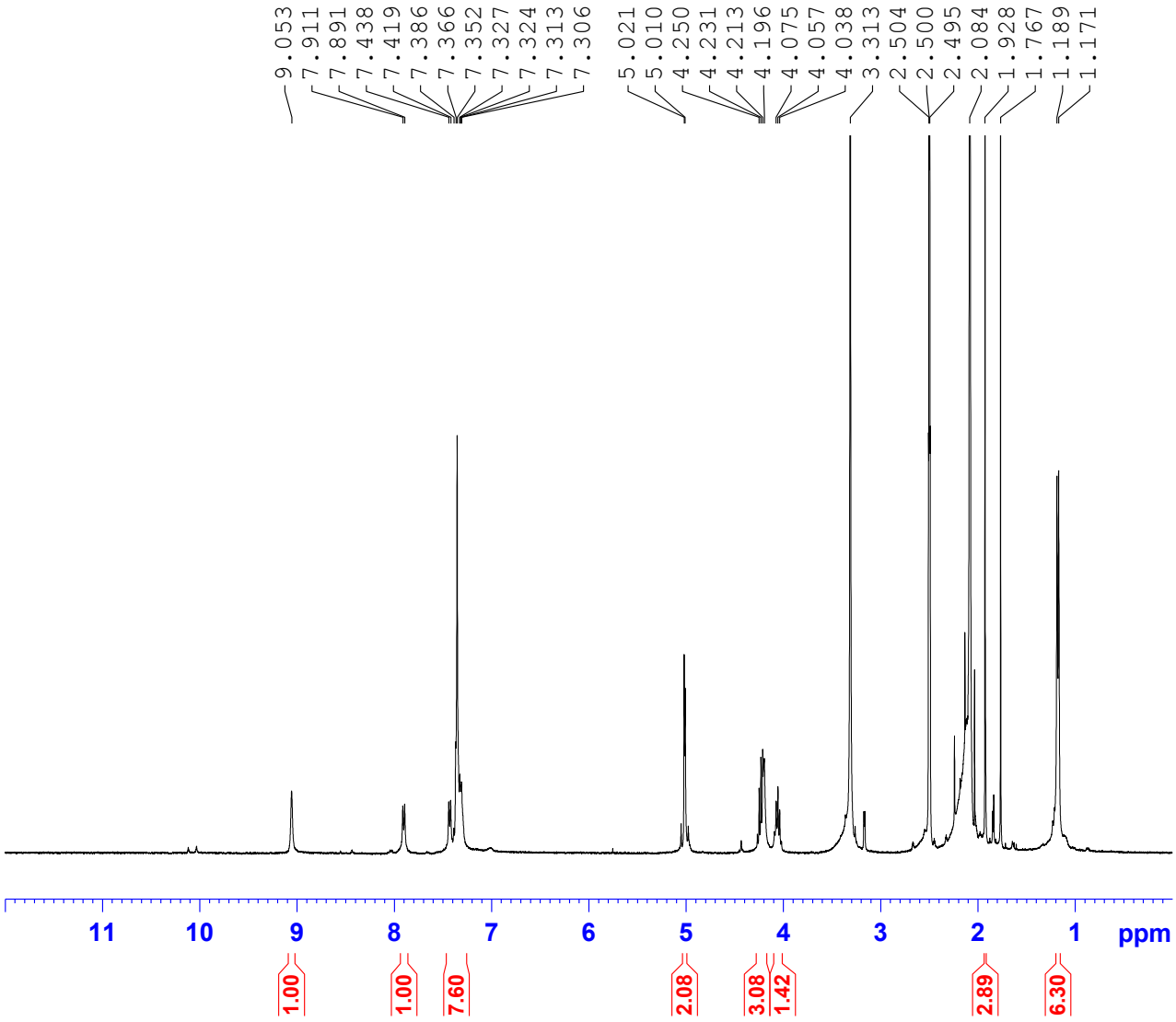
Z-Glu(OtBu)-Val-OMe



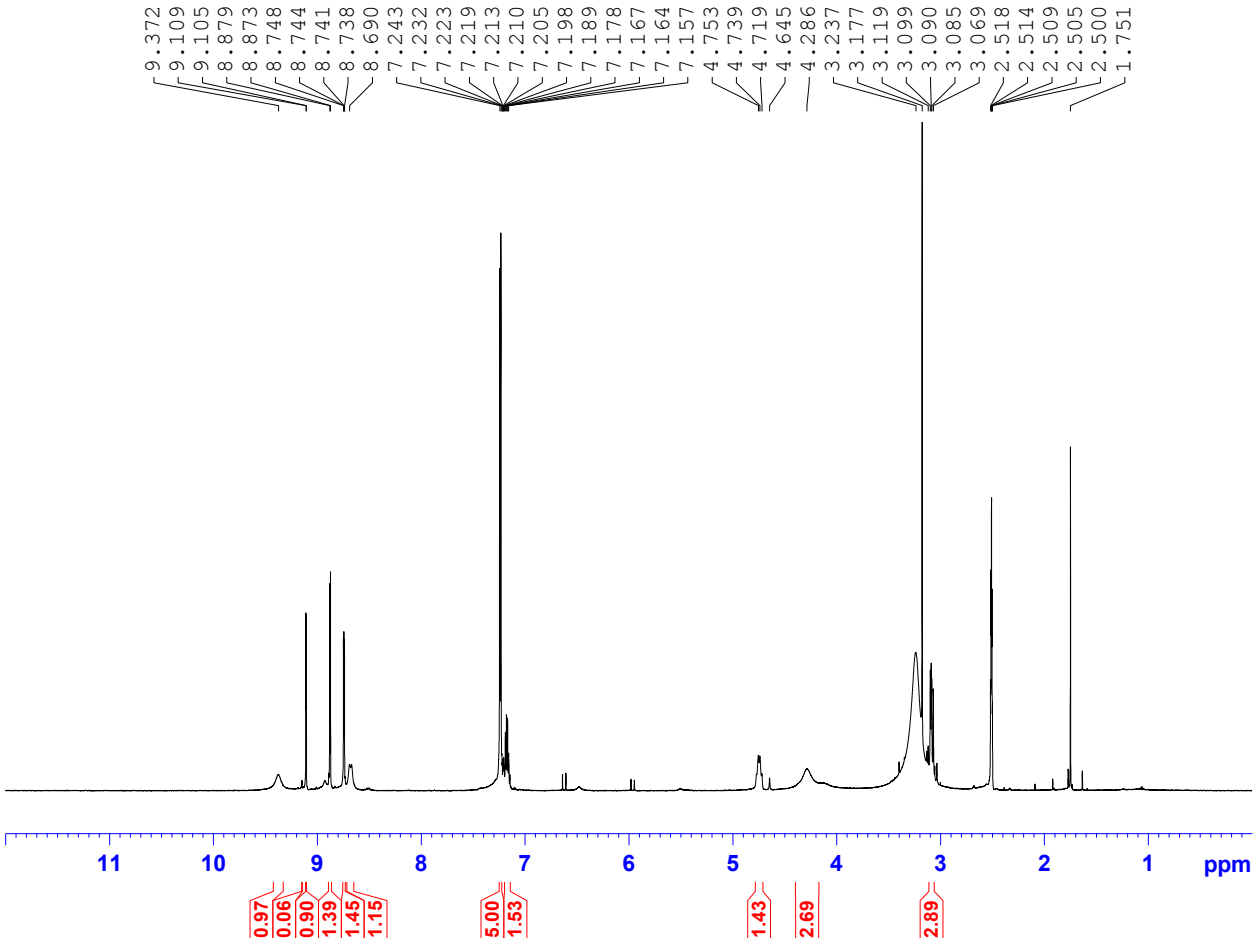
Z-Asp(OtBu)-Glu(OtBu)-Val-OMe



Z-Leu-Leu-NHNH$_2$



Z-Ala-Ala-NHNH$_2$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8.873
8.748
8.744
8.741
8.738
8.690
7.243
7.232
7.223
7.219
7.213
7.210
7.205
7.198
7.189
7.178
7.167
7.164
7.157
4.753
4.739
4.719
4.645
4.286
3.237
3.177
3.119
3.099
3.090
3.085
3.069
2.518
2.514
2.509
2.505
2.500
1.751
11
10
9
8
7
6
5
4
3
2
1
ppm
0.97
0.06
0.90
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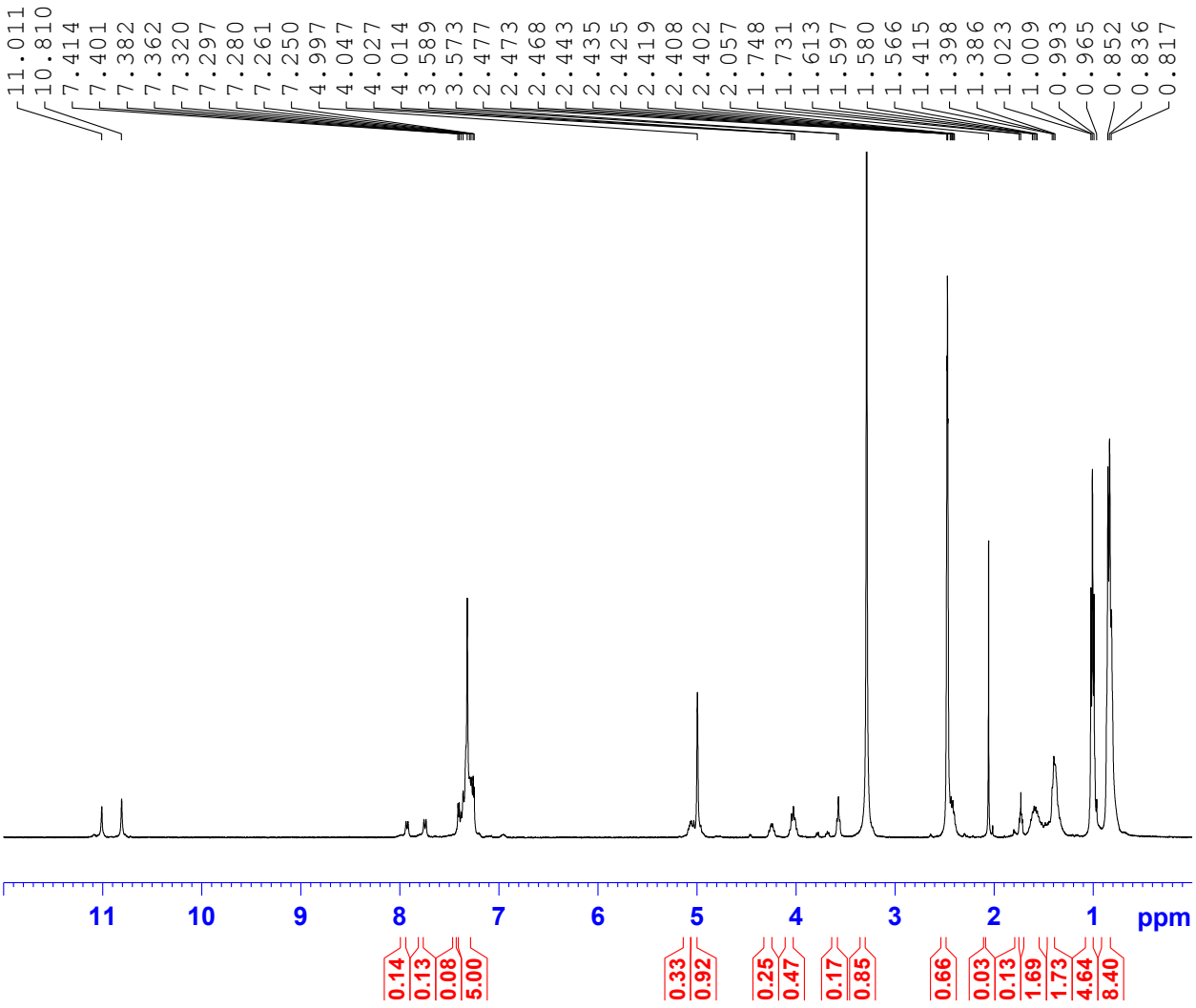
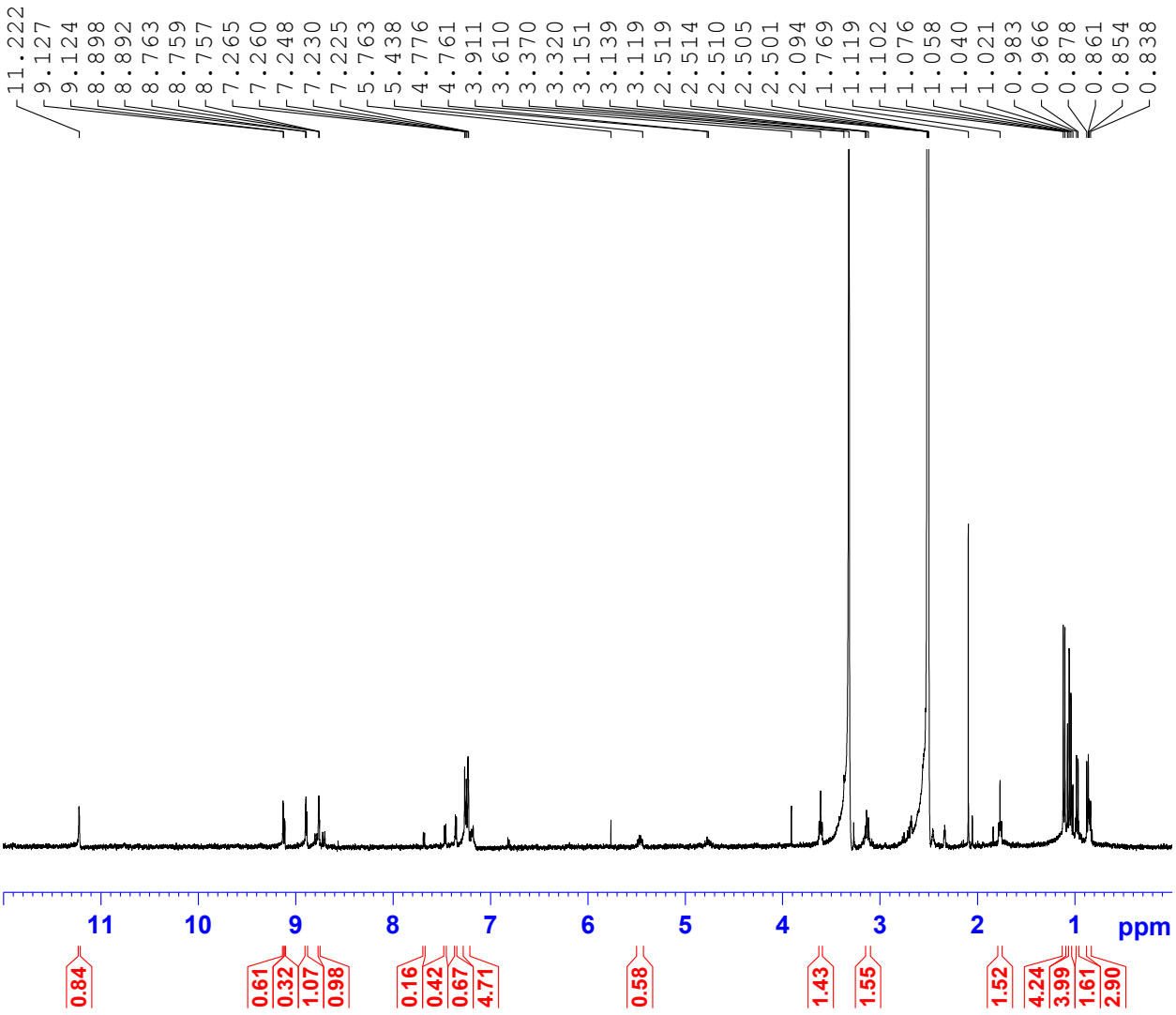
1.45
1.15
5.00
1.53
1.43
2.69
2.89

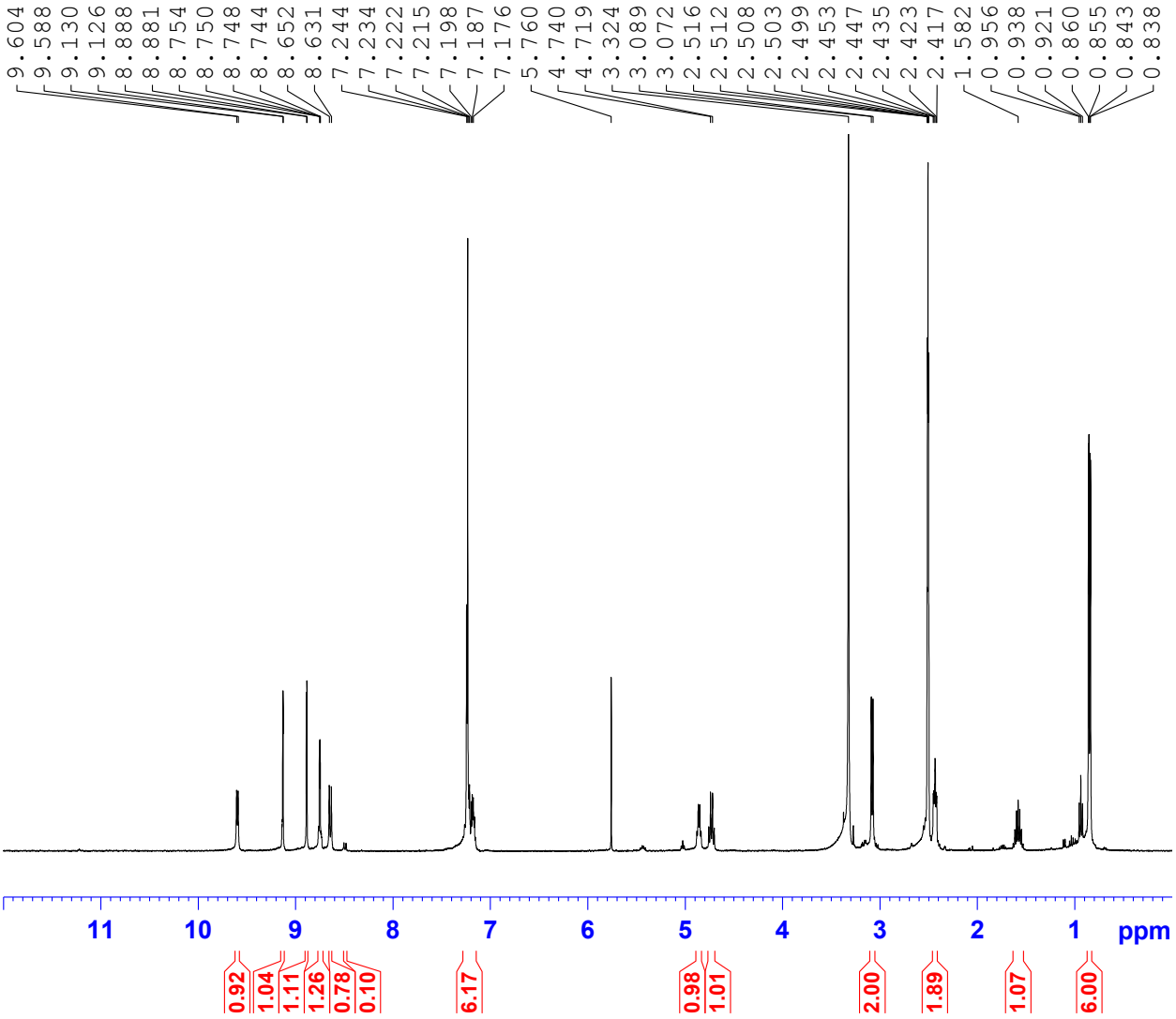
Pz-Phe-NH-NH_2



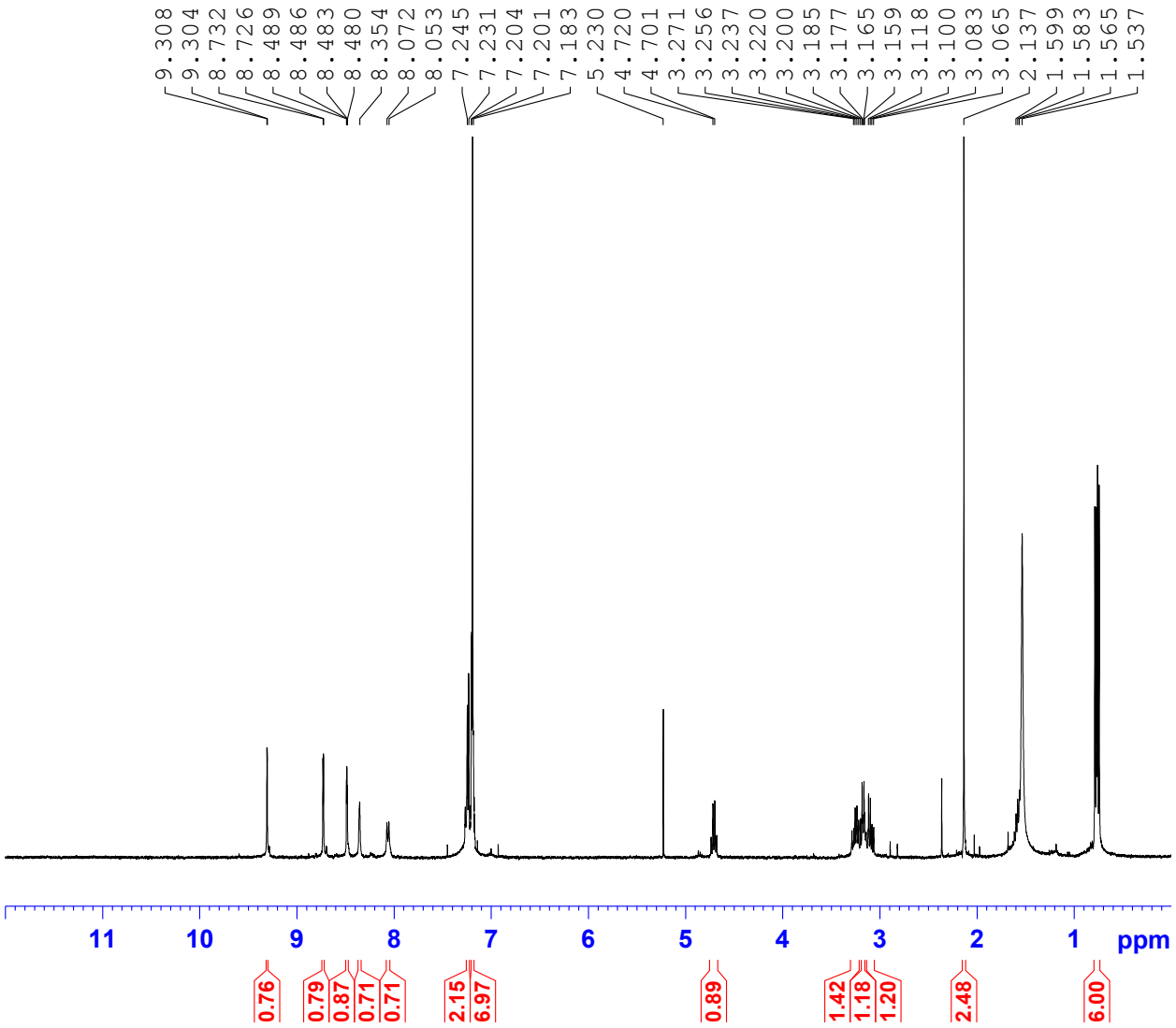
Z-Leu-Leu-NHN=CHCH(CH$_3$)$_2$



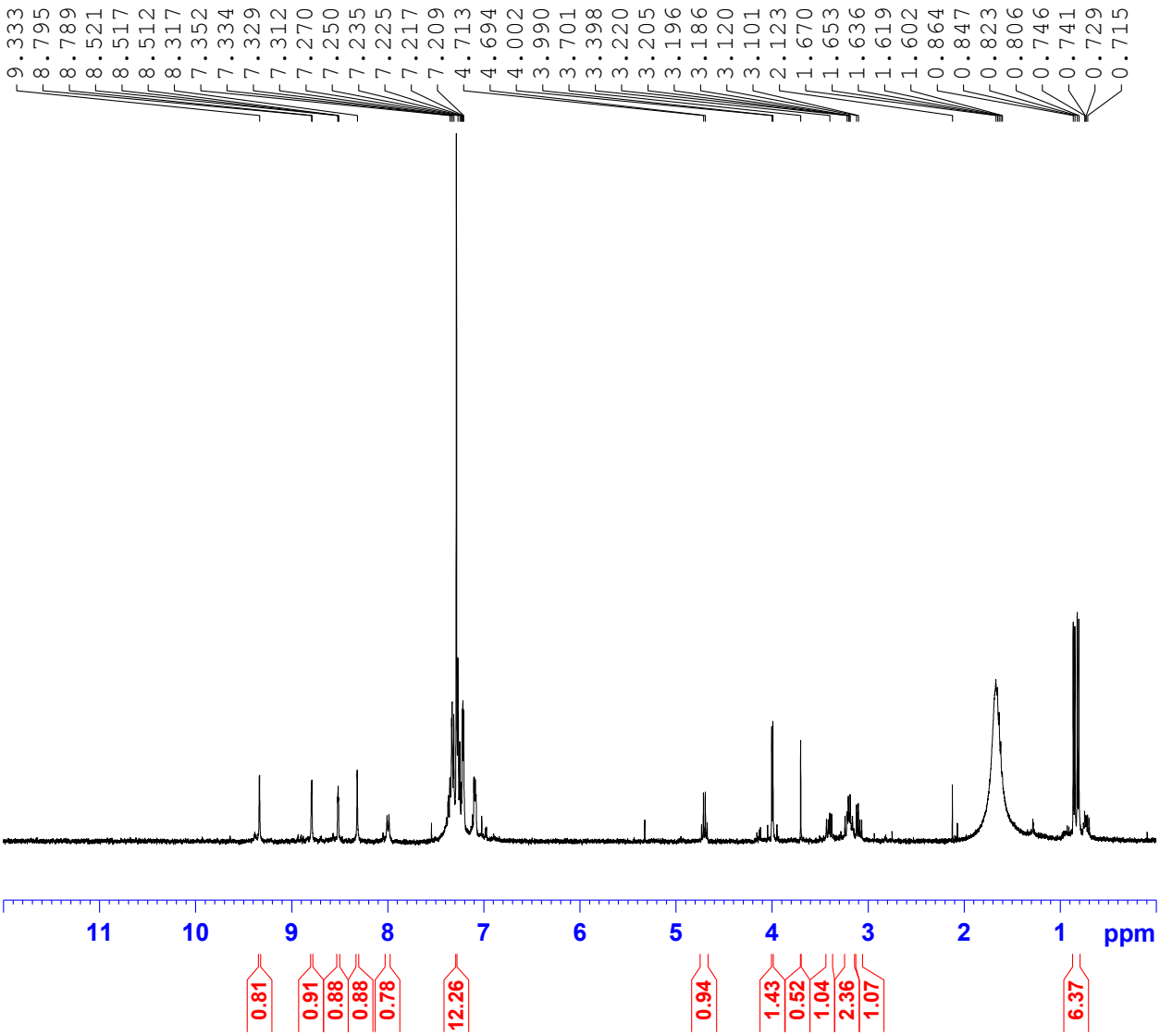
Pz-Phe-NHN=CHCH(CH$_3$)$_2$



Pz-Phe-NHNHCH$_2$CH(CH$_3$)$_2$



Pz-Phe-ALeu-COMe



Pz-Phe-ALeu-COBn